

Synergistic approaches to managing Gram-negative bacterial resistance

Edited by

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Synergistic approaches to managing Gram-negative bacterial resistance

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Editorial: Synergistic approaches to managing Gram-negative bacterial resistance

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KEYWORDS

Gram-negative bacteria, antimicrobial resistance, genomic studies, phenotypic studies, virulence, pathogen-host interaction

Editorial on the Research Topic

Synergistic approaches to managing Gram-negative bacterial resistance

Multiresistant Gram-negative bacterial pathogens pose a major threat to global health (Huttner et al., 2013; Macescic et al., 2025), and the continuous increase in antimicrobial resistance, coupled with the very limited introduction of new antibiotics, exacerbates this situation (Aslam et al., 2018). One effective way to counteract antimicrobial resistance is through containment measures that can be implemented at local, regional and international levels. However, such measures require the collection of epidemiological data to deduce appropriate strategies, which can be very time consuming. In addition to the development of new antimicrobial drugs, ongoing evaluation of further treatment strategies is crucial. Notably, phage therapy has re-gained importance in recent decades (Slopek et al., 1983), however, to successfully implement such alternatives, detailed knowledge of bacterial pathogenicity is essential. This includes genomic data, as well as knowledge regarding how pathogenic species interact with the host microbiome and immune system.

This Research Topic highlights current research in this field and emphasizes the threat of antibiotic resistance to public health. A major focus of this Research Topic was placed on *Klebsiella pneumoniae*. In this context, epidemiological data were collected on the distribution of various resistance genes, and fundamental research contributing to a better understanding of pathogenicity was presented (Li et al., Li et al., Zhong et al.). Of particular note, regarding the genetics of hypervirulent *K. pneumoniae*, Yan et al. demonstrated the importance of the *iucA* gene for the expression of the hypervirulent pathotype. Additionally, Klaper et al. proposed the existence of three *K. pneumoniae* pathovars (classical *K. pneumoniae*, ESBL-positive, and hypervirulent *K. pneumoniae*). Their research revealed that hypervirulent strains evade phagocytosis by macrophages and exhibit cytotoxic potential.

Further studies in this Research Topic investigated risk factors for infection with *Acinetobacter baumannii* in pediatric patients, as well as patients critically ill with COVID-19 (Wang et al., Ghamari et al.). An epidemiological study reported on patients in Lithuania who experienced invasive infection caused by *Neisseria meningitidis* (Ghamari et al.). Moreover, a new resistance mechanism to fosfomycin in *Morganella morganii* was identified and a new human pathogenic species of the genus *Stenotrophomonas* was introduced into the taxonomy (Zhang et al., Li et al.). The findings presented in this Research Topic highlight critical areas for research, hospital hygiene and public health initiatives, indicating which isolates and pathogens should become a primary focus in the future.

Author contributions

AZ: Writing – review & editing, Project administration. HH: Project administration, Writing – review & editing. BB: Project administration, Writing – review & editing. MC: Project administration, Writing – review & editing. PS: Writing – review & editing, Project administration, Supervision, Resources, Writing – original draft, Conceptualization.

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Klebsiella pneumoniae exhibiting a phenotypic hyper-splitting phenomenon including the formation of small colony variants

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In this study, we characterized a *Klebsiella pneumoniae* strain in a patient with shrapnel hip injury, which resulted in multiple phenotypic changes, including the formation of a small colony variant (SCV) phenotype. Although already described since the 1960s, there is little knowledge about SCV phenotypes in *Enterobacteriaceae*. The formation of SCVs has been recognized as a bacterial strategy to evade host immune responses and compromise the efficacy of antimicrobial therapies, leading to persistent and recurrent courses of infections. In this case, 14 isolates with different resisto- and morpho-types were distinguished from the patient's urine and tissue samples. Whole genome sequencing revealed that all isolates were clonally identical belonging to the *K. pneumoniae* high-risk sequence type 147. Subculturing the SCV colonies consistently resulted in the reappearance of the initial SCV phenotype and three stable normal-sized phenotypes with distinct morphological characteristics. Additionally, an increase in resistance was observed over time in isolates that shared the same colony appearance. Our findings highlight the complexity of bacterial behavior by revealing a case of phenotypic "hyper-splitting" in a *K. pneumoniae* SCV and its potential clinical significance.

KEYWORDS

Klebsiella pneumoniae, hyper-splitting, small colony variant, hypervirulence, multidrug resistance, in-host evolution, convergence

1 Introduction

Klebsiella pneumoniae, an opportunistic pathogen known for its ability to cause a wide range of nosocomial and community-acquired infections, has emerged as a significant public health threat due to its strain-specific, extensive arsenal of resistance and virulence factors (Wyres et al., 2020; Antimicrobial Resistance Collaborators, 2022). Infections caused by multi-, extensively-, and pandrug-resistant strains result in high mortality due to limited response to antibiotic therapy, which poses an increasing threat (Ventola, 2015; Navon-Venezia et al., 2017; Avgouleas et al., 2018). Apart from classic strains, a hypervirulent *K. pneumoniae* (hvKp) pathotype occurs and is characterized by invasive, often life-threatening and multiple site infection, characteristically in healthy patients from the general population (Russo and Marr, 2019). In addition, convergent types that successfully combine resistance and hypervirulence represent a “perfect storm” and have been increasingly reported in recent years (Heiden et al., 2020; Lan et al., 2021; Eger et al., 2022).

Beyond typical resistance mechanisms against various antimicrobials, functional resistance mechanisms have been elucidated that lead to antimicrobial treatment failure and foster the development of relapses and persistent infections (Ster et al., 2017). The formation of a biofilm matrix represents one of these mechanisms that facilitates antibiotic tolerance and the generation of bacterial persister cells (Ster et al., 2017). Interestingly, it has been demonstrated that a decrease in capsule biosynthesis, which is crucial for hypervirulent phenotypes, leads to increase *in vitro* biofilm formation and intracellular persistence (Ernst et al., 2020). Another non-classical mechanism leading to antibiotic tolerance is the formation of the small colony variant (SCV) phenotype. SCVs are subpopulations of bacteria that exhibit slow growth, reduced colony size, and altered phenotypic properties compared to their normal-growing counterparts, making them difficult to detect and treat effectively (Proctor et al., 2006; Becker, 2023). Their ability to evade the host’s immune surveillance and to undermine the effectiveness of antimicrobial interventions by host cell internalization results in intracellular persistence, which contributes significantly to the recurrence and chronicity of the infection (Tuchscherr et al., 2011; Kahl et al., 2016). Intracellular persistence have been shown for different human and animal cell types including endothelial and epithelial cells such as keratinocytes and osteoblasts (von Eiff et al., 2001; Strobel et al., 2016). Another pivotal attribute facilitating this phenomenon is their capability to modulate metabolic processes and virulence characteristics (Kriegeskorte et al., 2014; Proctor et al., 2014). Hypermutator SCVs characterized by higher mutation frequencies than wild-type strains and isolated especially from cystic fibrosis (CF) patients (Oliver et al., 2000; Prunier et al., 2003) have also been associated with antibiotic resistance (Schaaff et al., 2003; Besier et al., 2008) and biofilm formation (Morelli et al., 2015).

To date, research has focused on staphylococcal SCVs, while SCVs of Gram-negative bacteria have been investigated in only a few studies and case reports (Proctor et al., 2006). Although the formation of small colonies in *K. pneumoniae* has been noticed during resistance studies against cephalosporins in the mid-1960s (Benner et al., 1965), this issue has not received sufficient attention

and detailed research has not been conducted on this subject. The first clearly defined SCV of *K. pneumoniae* (SCV-Kp) in literature was obtained by *in vitro* exposure to gentamicin (Musher et al., 1979). SCV-Kp were also isolated from a patient treated with aminoglycoside antibiotics (Murray and Moellering, 1982). Smaller and non-mucoid colonies were obtained as a result of conjugation-induced mutation in the outer membrane protein of a hypervirulent *K. pneumoniae* isolate (Srinivasan et al., 2012). Another study showed that biofilm-forming *K. pneumoniae* developed heteroresistance to colistin by presenting slow-growing SCV-Kp (Silva et al., 2016).

Here, we report on *K. pneumoniae* isolates displaying 14 different resisto- and morpho-types obtained from an immunocompetent male patient, who had sustained a traumatic injury caused by shrapnel shell fragments. The isolates comprise an initial, mostly susceptible *K. pneumoniae* isolate with typical morphological characteristics isolated from the patient’s urinary specimen. From the urine and tissue samples, 13 additional phenotypes with different combinations of resistance and morphological characteristics including *K. pneumoniae* SCV phenotypes were isolated.

2 Materials and methods

2.1 Patient data

Sufficient information could not be obtained regarding the period from the patient’s first acetabular and femoral head shrapnel-caused war injury in Ukraine in March 2022, where he underwent hip prosthesis at an external center before his transfer to our orthopedic service in July 2022. Fracture-related joint infection treatment in our hospital continued through November 2022. The administration of antibiotics during this period included piperacillin/tazobactam from July to October, 2022, trimethoprim/sulfamethoxazole from July to August, 2022, cefiderocol from August to November, 2022, and colistin from October to November, 2022. Daptomycin was introduced into the treatment protocol starting from October 2022 upon detection of *Staphylococcus epidermidis* from intraoperatively obtained hip tissue samples and central venous catheter tip, and continued until the patient’s discharge. No other bacteria were isolated from clinical samples during this period. Subsequently, a planned course of post-discharge antibiotic suppression therapy with doxycycline for three months was initiated. The first identification of carbapenem-resistant *K. pneumoniae* (CRKP) occurred in July 2022, followed by the initial detection of SCV-Kp in September 2022. Therefore, we decided to aggregate and systematically assess the entirety of *K. pneumoniae* strains isolated from the patient.

2.2 Strain identification

The urine sample obtained from the patient was quantitatively inoculated onto a Columbia agar plate with 5% sheep blood (BD Diagnostics, Heidelberg, Germany) and a MacConkey II-Agar plate

(BD Diagnostics) using a 10 µl disposable sterile loop. The plates were then incubated for 48 hours. Tissue samples collected during surgery were inoculated onto Columbia agar plates with 5% sheep blood, MacConkey II-Agar plates, and Mueller Hinton Chocolate agar plates (all from BD Diagnostics). These plates were incubated under capnophilic conditions for up to seven days. The remaining tissue material was inoculated onto Schaedler agar and into BBL Fluid Thioglycollate media (both from BD Diagnostics) and incubated for up to 14 days under anaerobic and capnophilic conditions, respectively.

Preliminary characterization of each phenotype was grounded in colony morphology and minimal inhibitory concentration (MIC) results for antibiotics encompassed within the VITEK® 2 AST card specific to *Enterobacterales* (bioMérieux SA, Marcy l'Étoile, France) according to EUCAST criteria. All *K. pneumoniae* strains, isolated from various patient's specimens during the period from July to December 2022, were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) utilizing the MALDI Biotyper® sirius system (Bruker Daltonics, Bremen, Germany) with MBT Biotargets 96 (Bruker Daltonics). The presence of carbapenemase-encoding genes was verified by a loop-mediated isothermal amplification (LAMP)-based assay (eazypex®, AmplexDiagnostics, Gars-Bahnhof, Germany).

2.3 Characterization of the phenotypes

@Sequential subcultures of all phenotypic variants were carried out on various agar plates (including Columbia agar + 5% sheep blood, MacConkey agar from BD, and CHROMID® CPS® Elite agar from bioMérieux) to observe whether changes in colony morphology occurred and SCVs remained stable, followed by meticulous analysis of generated phenotypic profiles.

In order to determine colony sizes, each phenotype was inoculated onto 5% sheep blood agar plates in triplicate on different days. After overnight incubation at $35 \pm 1^\circ\text{C}$ in ambient air, the diameters of ten colonies of each phenotype were measured and mean values were determined. Additionally, colony morphology in different phenotypes was assessed using the stereo zoom microscope Axio Zoom.V16, equipped with the objective Plan Z 1.0x/0.25 and the Axiocam 305 camera (Zeiss, Oberkochen, Germany). After Gram staining, single cells from different phenotypes were observed in transmission light by the Axio Imager.Z2m microscope with the oil immersion objective Plan-APOCHROMAT 100x/1.4 and Axiocam 305 camera (Zeiss).

2.4 Antimicrobial susceptibility testing

In addition to the initial VITEK® 2 AST, the MICs of a standardized set of antibiotics (Table 1) were determined by the broth microdilution (BMD) method using cation-adjusted Mueller–Hinton broth (CAMHB; Micronaut-S 96-well microtiter plates, Merlin, Bornheim-Hersel, Germany), and for cefiderocol using iron-depleted CAMHB (UMIC®, Merlin, Bornheim-Hersel, Germany), as recommended by ISO 20776-1, the European

Committee on Antimicrobial Susceptibility Testing (EUCAST), and the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018; Standardization, 2019; EUCAST, 2023b). The results were observed following 18 ± 2 hours of incubation at $35 \pm 1^\circ\text{C}$ in ambient air. All tests were conducted in triplicate on different days, and median MIC values were computed for analysis. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control (QC) strains, and their results were within the QC range throughout the study. EUCAST Clinical Breakpoint Tables v. 13.1 were used for MIC interpretation (EUCAST, 2023a).

2.5 DNA isolation and sequencing

After overnight growth on blood agar plates at 37°C , ten colonies were randomly selected and suspended in 1.5 mL tubes (Carl Roth, Karlsruhe, Germany) with 1 mL of phosphate buffered saline. Total DNA was extracted using the MasterPure DNA Purification kit for Blood, v. 2 (Lucigen, Middleton, WI, USA) according to the manufacturer's instructions. Quantification of isolated DNA was performed with the Qubit 4 fluorometer and the dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was sent to SeqCenter (Pittsburgh, PA, USA), where sample library preparation using the Illumina DNA Prep kit and IDT 10bp UDI indices was performed. Subsequently, libraries were sequenced on an Illumina NextSeq 2000, producing 2x151bp reads. Demultiplexing, quality control and adapter trimming at the sequencing center was performed with bcl-convert v. 3.9.3 (https://support-docs.illumina.com/SW/BCL_Convert/Content/SW/FrontPages/BCL_Convert.htm).

2.6 Assembly and genomic characterization

We employed a custom assembly and polishing pipeline to assemble raw sequencing reads to contigs. This pipeline consists of four parts, namely trimming (BBduk from BBTools v. 38.98 [<https://sourceforge.net/projects/bbmap/>], quality control (FastQC v. 0.11.9 [<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>]), assembly (shovill v. 1.1.0 [<https://github.com/tseemann/shovill>]) with SPAdes v. 3.15.5 (Prjibelski et al., 2020), and polishing (BWA-MEM2 v. 2.2.1 (Vasimuddin et al., 2019), Polypolish v. 0.5.0 (Wick and Holt, 2022)). Genotyping was performed with Kleborate v. 2.2.0 (Lam et al., 2021) and Kaptive (Wyres et al., 2016; Lam et al., 2022).

2.7 Confirmation of clonality

Trimmed sequencing reads of all isolates were mapped against isolate 1-A with snippy v. 4.6.0 (<https://github.com/tseemann/snippy>) and the SNP distance matrix calculated with snp-dists v. 0.8.2 (<https://github.com/tseemann/snp-dists>).

TABLE 1 Colony morphology and antimicrobial susceptibility characteristics of the 14 phenotypes of the *Klebsiella pneumoniae* strain.

14 phenotypes of the <i>K. pneumoniae</i> strain					Median minimum inhibitory concentrations (MICs), µg/mL ^{a,b}																	Presence of <i>bla</i> genes	
Isolate number	Specimen	Date	Colony morphology		PIP	TZP	TEM	FDC	CTX	CAZ	CZA	C/T	IPM	MEM	CIP	LVX	AMK	TGC	CHL	CST	FOF		SXT
			Color	Surface																			
1-A	Urine	Jul 22	Whitish	Glistening, smooth	≤8	≤4	≤32	≤0.03	≤1	≤1	≤1	≤1	≤1	≤0.125	>2	>2	≤4	0.5	16	≤1	>128	≤1	<i>bla</i> _{SHV-11}
1-B			Grey	Glistening, smooth	≤8	≤4	≤32	≤0.03	≤1	≤1	≤1	≤1	≤1	≤0.125	>2	>2	≤4	0.5	16	≤1	>128	≤1	
2-A	Intra-operatively obtained hip tissue	Jul 22	Whitish	Glistening, smooth	>16	>64	>128	1	>2	>128	>16	>8	>8	128	>2	>2	8	0.5	>16	≤1	>128	≤1	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{OXA-48} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{NDM-1} , <i>bla</i> _{SHV-11}
2-B			Grey	Glistening, smooth	>16	8	≤32	0.06	≤1	≤1	≤1	≤1	≤1	≤0.125	>2	>2	8	≤0.25	≤8	≤1	>128	≤1	<i>bla</i> _{OXA-9} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{SHV-11}
3-A	Intra-operatively obtained hip tissue	Aug 22	Whitish	Glistening, smooth	>16	>64	>128	1	>2	>128	>16	>8	>8	128	>2	>2	8	≤0.25	>16	≤1	>128	≤1	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{OXA-48} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{NDM-1} , <i>bla</i> _{SHV-11}
3-B			Grey	Glistening, smooth	>16	>64	>128	2	>2	>128	>16	>8	>8	128	>2	>2	8	≤0.25	>16	≤1	>128	≤1	
4-A	Intra-operatively obtained hip tissue	Sep 22	Small colony variant		>16	>64	>128	0.25	>2	>128	>16	>8	>8	128	>2	>2	32	0.5	>16	≤1	>128	>4	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{OXA-48} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{NDM-1} , <i>bla</i> _{SHV-11}
4-B			Whitish	Glistening, smooth	>16	64	>128	0.125	2	≤1	≤1	≤1	8	16	>2	>2	8	0.5	>16	≤1	>128	2	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{OXA-48} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{CTX-M-15} ^c , <i>bla</i> _{NDM-1} , <i>bla</i> _{SHV-11}
4-C			Grey	Glistening, smooth	>16	>64	>128	1	>2	>128	>16	>8	>8	128	>2	>2	8	0.5	>16	≤1	>128	4	
4-D			Grey	Dry, rough	>16	>64	>128	2	>2	>128	>16	>8	>8	64	>2	>2	8	0.5	>16	≤1	>128	≤1	
5-A	Intra-operatively obtained hip tissue	Sep 22	Small colony variant		>16	>64	>128	0.25	>2	>128	>16	>8	>8	>128	>2	>2	32	0.5	>16	≤1	>128	>4	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{OXA-48} , <i>bla</i> _{TEM-1D} , <i>bla</i> _{CTX-M-15}
5-B			Whitish	Glistening, smooth	>16	>64	>128	2	>2	>128	>16	>8	>8	128	>2	>2	8	0.5	>16	≤1	>128	4	
5-C			Grey		>16	>64	>128	2	>2	>128	>16	>8	>8	128	>2	>2	8	0.5	>16	≤1	>128	4	

(Continued)

TABLE 1 Continued

14 phenotypes of the <i>K. pneumoniae</i> strain				Median minimum inhibitory concentrations (MICs), µg/mL ^{a,b}																	Presence of <i>bla</i> genes		
Isolate number	Specimen	Date	Colony morphology		PIP	TZP	TEM	FDC	CTX	CAZ	CZA	C/T	IPM	MEM	CIP	LVX	AMK	TGC	CHL	CST	FOF	SXT	
			Surface	Color																			
5-D				Glistening, smooth																			
			Grey	Dry, rough	>16	>64	>128	1	>2	>128	>16	>8	>8	64	>2	>2	8	0.5	>16	≤1	>128	4	

^aAbbreviations of antibacterial agents: PIP, piperacillin; TZP, piperacillin-tazobactam; TEM, temocillin; FDC, cefiderocol; CTX, cefotaxime; CAZ, ceftazidime; C/T, ceftiozane-tazobactam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; TGC, tigecycline; CHL, chloramphenicol; CST, colistin; FOF, fosfomicin; SXT, trimethoprim-sulfamethoxazole.

^bCells with red background, MIC test result categorized as resistant; cells with green background, MIC test result categorized as susceptible (according to EUCAST Breakpoint tables for interpretation of MICs and zone diameters, Version 13.1, 2023);

^cIn isolate 4-B, *bla*_{CTX-M-15} and *bla*_{NDM-1} genes were detected by WGS, but not by LAMP after sub-cultivation for antimicrobial susceptibility testing.

3 Results

Overall, 14 distinct phenotypes were determined (Table 1). From the urine, two phenotypes (1-A and 1-B) exhibiting a normal colony size and glistening surface but differing in the color of their colonies displaying whitish or grey colonies, were isolated. All other phenotypes (n = 12) were isolated from tissue specimens. Strains numbered 1-A, 2-A, 3-A, 4-B, 5-B, numbered 1-B, 2-B, 3-B, 4-C, 5-C, and numbered 4-D, 5-D, displayed identical morphological attributes each, distinguished by whitish, glistening, and smooth (Figure 1B), grey, glistening, and smooth (Figure 1C), and grey, dry, and rough colonies (Figure 1D), respectively (Supplementary Figure S1). These strains revealed a normal colony size of 2.4 mm on average (range, 1 – 5.5 mm). The isolates displaying the SCV phenotype, numbered 4-A and 5-A, exhibited similar morphological characteristics, and colony sizes were smaller than 0.5 mm (Figure 1, Supplementary Figure S1). No discernible variation in terms of colony clustering was observed among the various agar plates. There were no obvious differences in size or shape of cells between different phenotypes except bacteria from grey, dry, rough phenotype 5-D, in which cells were clearly elongated (Supplementary Figure S2).

Initially, largely antibiotic-susceptible *K. pneumoniae* phenotypes exhibiting whitish and grey colony morphologies on Columbia agar plates were isolated from the urine sample. Following antibiotic treatment, MDR *K. pneumoniae* strains displaying the normal colony size were isolated from tissue samples, again characterized by subsequent whitish or grey colony formations. Subsequently, SCVs of *K. pneumoniae* were isolated from tissue samples. Subcultivation of different SCV colonies consistently yielded a division into four distinct colony morphotypes including one SCV phenotype that resembled the initial SCV, along with three normal-sized phenotypes distinguished by variations in colony color and visual attributes. While normal-sized phenotypes exhibited stability following each round of re-cultivation, SCV isolates displayed instability and recurrently diverged into the four phenotypes described above. We have designated the emergence of these multiple phenotypes as “hyper-splitting”. Despite minor variations in MIC values, these “hyper-splitting” phenotypes exhibited multidrug resistance (Table 1).

Except for isolates 1-A and 1-B, all isolates were resistant to the tested carbapenems. Initially, during routine diagnosis, isolate 2-B was found to be carbapenem-resistant by VITEK® 2 AST, and to harbor *bla*_{OXA-48} gene by LAMP. After subcultivation of this isolate for MIC determination, this resistance disappeared and the isolate became susceptible to all tested beta-lactam antibiotics except piperacillin. We assume that a mobile genetic element harboring *bla*_{OXA-48} gene was lost upon subcultivation. Only isolates 1-A and 1-B were susceptible to piperacillin, and only isolate 4-B was not resistant to the cephalosporins tested. Interestingly, only isolates 4-A and 5-A, which demonstrated the SCV phenotype, were resistant to amikacin and trimethoprim-sulfamethoxazole. Another remarkable finding was the observed increase in the MIC values of cefiderocol and trimethoprim-sulfamethoxazole over time (Table 1).

Whole-genome sequence (WGS) analysis revealed that all isolates belonged to sequence type (ST) 147. Lipopolysaccharide antigen (O) loci were O1/O2v1 and capsule biosynthesis (KL) loci were KL64 for all isolates except isolate 4-D, which could not be

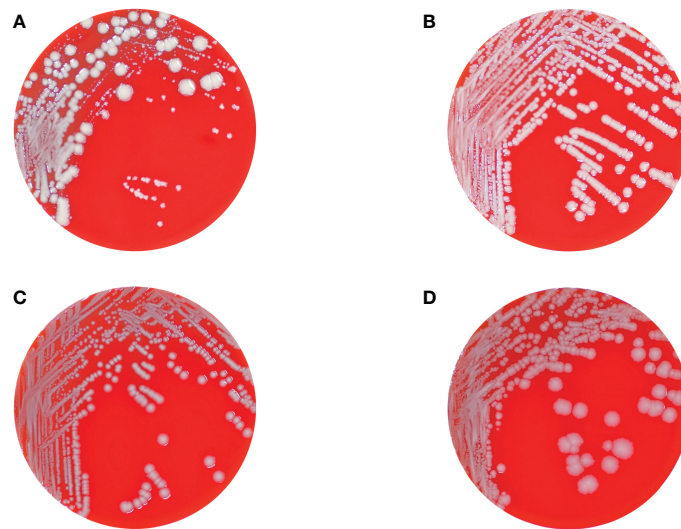


FIGURE 1

Columbia blood agar plates showing the different colonial morphotypes of the *K. pneumoniae* isolates comprising regular sized colonies (wild-type) with glistening whitish (B) and grey (C), and dry and rough grey colonies (D), respectively, as well as tiny grey and whitish colonies displaying the SCV phenotype (A). Panel (A) also shows the hyper-splitting phenomenon of the SCV phenotype into the colony morphotypes shown in panels (B–D).

assigned, as it missed most genes of this locus. Isolates 1-A, 1-B and 2-B showed lower Kleborate resistance score than the other isolates (resistance: 0 vs. 2). The resistance score of 0 indicates that the isolate(s) did not carry any genes for extended-spectrum beta-lactamases (ESBL) or carbapenemases and a score of 2 correlated with the presence of carbapenemase genes without colistin resistance genes (Lam et al., 2021). In accordance with the resistance scores, we detected several beta-lactamase genes, such as *bla*_{SHV-11}, *bla*_{TEM-1} and *bla*_{OXA-9}, ESBL genes, such as *bla*_{CTX-M-15} and *bla*_{OXA-1}, and the carbapenemase genes *bla*_{NDM-1} and *bla*_{OXA-48}. *bla*_{SHV-11} was found in all isolates whereas *bla*_{TEM-1} and *bla*_{OXA-9} were present in all isolates except 1-A and 1-B. However, *bla*_{CTX-M-15} was not found in isolate 4-A. In isolate 4-B, *bla*_{CTX-M-15} and *bla*_{NDM-1} genes were initially detected by WGS, however, after sub-cultivation, a discrepancy between AST and WGS results was observed. Re-testing by LAMP at this later time point revealed the loss of both genes (Table 1). Genes associated with sulphonamide (*sul1*) and chloramphenicol (*catB3*) resistance were also detected in all isolates except 1-A, 1-B and 2-B. Note that we did not detect any common cefiderocol resistance genes.

The isolates exhibited clonality as emphasized by the low number of SNPs among them (Supplementary Tables S1, S2). Especially isolates from the same time point showed no difference in the core genome alignment (5,360,988 bp) with the exception of 2-A and 2-B (six SNPs) and 5-D (one additional SNP compared to 5-A–C). The largest distance with 17 SNPs was between 2-A and 5-D (Supplementary Table S1).

4 Discussion

When evaluating the results, we can roughly identify three distinct outcomes. The first significant observation concerns the

emergence of resistance development chronologically within a *K. pneumoniae* strain, originating from a patient subjected to continuous, uninterrupted antibiotic intervention. This scenario promptly elicits contemplation of the subject concerning within-host adaptive evolution of bacteria. In fact, in-host resistance evolution, either due to plasmid mediation or chromosome mutations, has been observed even shortly after the initiation of antimicrobial treatment (Jin et al., 2021).

The second notable observation in our study is the occurrence of SCVs from patient specimens following the detection of normalized morphotypes. SCVs demonstrate remarkable abilities to invade and persist within host cells, thus evading the surveillance mechanisms of the immune system (Tuchscher et al., 2020). The existence of SCVs, mostly observed in *Staphylococcus* spp., has been documented since the onset of the 20th century and has gained increasing attention due to its potential implications for both clinical and basic research (Jacobsen, 1910; Proctor et al., 2006). Regarding the SCVs of Gram-negative bacteria, studies have particularly focused on *Burkholderia* and *Pseudomonas* spp. isolated from CF patients (Oliver et al., 2000; Haussler et al., 2003a, Haussler et al., 2003b). However, there are only sparse data on the occurrence of SCV in *Klebsiella* spp (Benner et al., 1965; Musher et al., 1979; Murray and Moellering, 1982; Srinivasan et al., 2012; Silva et al., 2016).

Basically, SCVs have been determined as a subpopulation characterized by their distinct phenotypic properties, such as atypical colony morphologies including the reduced colony size (Proctor et al., 1995). Their decreased growth rate is thought to contribute to their inherent resistance, given that the decelerated growth dynamics potentially hinder the effectiveness of antibiotics geared towards rapidly proliferating cell populations (Proctor and von Humboldt, 1998). Furthermore, this phenomenon concurrently signifies decreased metabolic activity, which may engender

modifications in cell wall permeability, drug uptake, or the modulation of efflux pump expression (Mitsuyama et al., 1997).

For electron transport chain-defective staphylococcal SCVs, lower efficacy of aminoglycosides known to be taken up through electrical potential across the cytoplasmic membrane ($\Delta\Psi$) was demonstrated, which is attributable to low $\Delta\Psi$ (Baumert et al., 2002). These alterations could collectively contribute to enhancing resistance patterns. In this study, we observed an increase in the MIC values of amikacin, cefiderocol, and trimethoprim-sulfamethoxazole in the isolates recovered over time. This MIC increase was especially pronounced for amikacin in SCV phenotypes. Moreover, most antibiotics penetrate into host cells poorly, so the concentrations required to kill intracellularly persistent SCVs cannot be achieved (Proctor et al., 2006).

SCVs, known for their inducible formation through *in vitro* processes involving various agents, including antibiotics (Benner et al., 1965), have exhibited a propensity for increased persistence and adaptability when confronted with challenging environments (Li et al., 2016). An enhanced ability to form biofilms on biotic and abiotic surfaces has been shown for SCVs of different bacterial species (Haussler et al., 2003b; Webb et al., 2004; Al Laham et al., 2007; Allegrucci and Sauer, 2007; Millette et al., 2023). The substantial implication of SCVs extends to their involvement in biofilm development, as biofilms effectively shield bacteria from harsh host environments, thereby complicating the elucidation of drug resistance mechanisms within biofilm structures (Craft et al., 2019). Biofilms not only confer protection against host immune defenses but also serve as reservoirs for persistent infections and recurrent episodes (Mirani et al., 2015). The impact of SCV phenotype on biofilm formation in *Klebsiella* remains to be elucidated in further studies.

Furthermore, the emergence of SCVs could plausibly be due to selection pressure from antibiotic regimens or other host-associated factors, e.g., host cationic peptides. Consistent with the case that was the subject of our study, the higher frequency of SCVs in isolates from chronic and recurrent infections compared to acute infections suggests a potential role for these variants in evading host immune responses and antimicrobial treatments (Proctor et al., 2006). In the context of our study, the emergence of SCVs after the initiation of cefiderocol treatment while already undergoing antibiotic therapy could be construed as a form of *in vivo* or *in host* induction.

The third noteworthy finding from our study underscores the inherent instability of SCVs. This dynamic interplay between stable and unstable SCVs is still poorly understood and its elucidation may contribute to a deeper understanding of their role in infection in general and persistence phenomena in particular (Becker et al., 2006). Despite comprehensive explorations largely focusing on staphylococci, a lack of investigations concerning *Klebsiella* spp. persists, and requires attention.

The observed instability among SCVs, combined with distinct antibiotic susceptibility profiles across phenotypes, increases the significance of investigating SCV plasticity (Proctor et al., 1995). Stable SCVs represent a long-term adaptation strategy, whereas their unstable counterparts may arise as stress-induced variants that result from rapid adaptation to fluctuating environments

(Tuchscherr et al., 2010, Tuchscherr et al., 2011, Tuchscherr et al., 2015). This inherent instability potentially serves as a mechanism for evading host immune responses and circumventing antibiotic interventions (Tuchscherr et al., 2015). Furthermore, the involvement of epigenetic modifications, including alterations in DNA methylation patterns, could significantly influence SCV stability (Guerillot et al., 2019). In addition, regulatory systems, such as two-component systems and quorum sensing, play a crucial role in SCV formation by modulating bacterial behavior and adaptation. Disruption or dysregulation of these systems could lead to the emergence of SCVs with altered phenotypic properties (Pader et al., 2014). Due to instability, slow-growing SCVs may generate mutants that exhibit a faster growth rate than usual (Brandis et al., 2017). In instances of reversion to the wild type, rapidly growing mutant revertants may demonstrate either the loss or preservation of antibiotic resistance (Brandis et al., 2017).

A high mutation rate might favor the emergence of SCVs (Schaaff et al., 2003) and also explain the emergence of antibiotic resistance as a result of antibiotic selective pressure and the adaptation of hypermutable strains in patients, especially CF patients (Prunier et al., 2003). CF-like chronic infections have been shown to specifically contribute to the development of bacterial mutations (Smith et al., 2006). Hypermutation could result in a subpopulation of bacteria that temporarily does not grow, thus leading to persistence (Witzany et al., 2022). Additionally, an increase in the prevalence of mutator bacterial strains with deficient DNA mismatch repair (MMR) system has been detected in CF patients, who are used as a reservoir for mutation (Mena et al., 2008). To our best knowledge, we were unable to identify any instance in the available literature wherein a solitary SCV colony has given rise to four distinct colonies exhibiting disparate morphologies. Accordingly, we suggest the designation “phenotypic hyper-splitting” for this distinctive phenomenon.

We described in this study unprecedented phenotypic attributes and primarily focused on *in vitro* experiments. Therefore, the clinical relevance of our findings necessitates validation through animal models and clinical sample analyses. In this context, macrophage and neutrophil assays would be valuable for assessing both the extent of immune response and the presence of persistent cells. Moreover, the determination of the auxotrophism (Kriegeskorte et al., 2014; Becker, 2023) of *K. pneumoniae* SCVs and of the molecular mechanisms that drive SCV formation and the resulting antibiotic resistance in this species require further investigation. Integrating a comprehensive range of approaches encompassing genomics, transcriptomics, and proteomics, the utilization of experimental evolutionary models can yield valuable insights into the genetic determinants and regulatory networks orchestrating SCV phenotypes.

The genomic analysis conducted in this study has revealed clonality among all 14 isolates. Further exploration is warranted to uncover the intricate molecular mechanisms underlying phenotypic hyper-splitting and to elucidate the potential pathogenic implications of this phenomenon. To better understand the formation of the SCV phenotype especially in Gram-negative pathogens, efforts need to be intensified (i) to improve the

detection and characterization of SCVs recovered from clinical samples and (ii) to elucidate their clinical impact.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ebi.ac.uk/ena>, PRJEB71325.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

ED: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization. KSy: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. SH: Data curation, Formal analysis, Software, Visualization, Writing – review & editing. EE: Writing – review & editing, Investigation, Data curation. GW: Validation, Writing – review & editing. RP: Validation, Writing – review & editing. JB: Validation, Writing – review & editing. EI: Writing – review & editing, Validation, Project administration, Methodology, Conceptualization. KSc: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. KB: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1372704/full#supplementary-material>

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Characterizing carbapenemase-producing *Escherichia coli* isolates from Spain: high genetic heterogeneity and wide geographical spread

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Introduction: Carbapenemase-Producing *Escherichia coli* (CP-Eco) isolates, though less prevalent than other CP-Enterobacterales, have the capacity to rapidly disseminate antibiotic resistance genes (ARGs) and cause serious difficult-to-treat infections. The aim of this study is phenotypically and genotypically characterizing CP-Eco isolates collected from Spain to better understand their resistance mechanisms and population structure.

Methods: Ninety representative isolates received from 2015 to 2020 from 25 provinces and 59 hospitals Spanish hospitals were included. Antibiotic susceptibility was determined according to EUCAST guidelines and whole-genome sequencing was performed. Antibiotic resistance and virulence-associated genes, phylogeny and population structure, and carbapenemase genes-carrying plasmids were analyzed.

Results and discussion: The 90 CP-Eco isolates were highly polyclonal, where the most prevalent was ST131, detected in 14 (15.6%) of the isolates. The carbapenemase genes detected were *bla*_{OXA-48} (45.6%), *bla*_{VIM-1} (23.3%), *bla*_{NDM-1} (7.8%), *bla*_{KPC-3} (6.7%), and *bla*_{NDM-5} (6.7%). Forty (44.4%) were resistant to 6 or more antibiotic groups and the most active antibiotics were colistin (98.9%), plazomicin (92.2%) and cefiderocol (92.2%). Four of the seven

cefiderocol-resistant isolates belonged to ST167 and six harbored *bla*_{NDM}. Five of the plazomicin-resistant isolates harbored *rmt*. IncL plasmids were the most frequent (45.7%) and eight of these harbored *bla*_{VIM-1}. *bla*_{OXA-48} was found in IncF plasmids in eight isolates. Metallo- β -lactamases were more frequent in isolates with resistance to six or more antibiotic groups, with their genes often present on the same plasmid/integron. ST131 isolates were associated with *sat* and *pap* virulence genes. This study highlights the genetic versatility of CP-Eco and its potential to disseminate ARGs and cause community and nosocomial infections.

KEYWORDS

carbapenemases, *Escherichia coli*, antibiotic resistance, virulence factor genes, whole-genome sequencing, sequence type, plasmids

1 Introduction

Antibiotic-resistant *Escherichia coli* is one of the principal pathogens that can cause community-acquired and nosocomial infections with increased morbidity and mortality rates (Hu et al., 2022). They are considered as one of the principal causes of Urinary Tract Infections (UTIs), bacteremia, and Intra-Abdominal Infections (IAI) (Balasubramanian et al., 2023). *E. coli* have a heightened ability to acquire Antibiotic-Resistance Genes (ARGs), such as the *bla*_{CTX-M-15} Extended Spectrum β -Lactamase (ESBL), and rapidly disseminate them throughout the community (González et al., 2020). Carbapenemase-producing *Escherichia coli* (CP-Eco), though not as frequently isolated in the clinical setting as compared to other CP-producing Enterobacterales (such as *Klebsiella pneumoniae* and *Enterobacter cloacae* complex), is especially worrying. This is because they are increasing in prevalence (Cañada-García et al., 2022), creating a concern that they would be able to spread carbapenemase genes in the community in a similar manner as it was observed for ESBLs (González et al., 2020). Moreover, these isolates are typically resistant to several other antibiotics, making their related infections difficult to treat (Boutzoukas et al., 2023).

All main carbapenemase families have been detected in CP-Eco (Grundmann et al., 2017), in addition to a wide range of virulence determinants that negatively affect clinical outcome (Čurová et al., 2020). All this led to the declaration by the World Health Organization that CP-Eco are a critical priority issue (Tacconelli et al., 2018). Globally, antibiotic-resistant *E. coli* has the highest incidence of hospital-acquired infections in high-to-mid income countries, causing three to twenty-five million infections per year (Balasubramanian et al., 2023). In Europe, the median number of infections caused by CP-Eco was 2,619 in 2015, with a median of 141 attributable deaths (Cassini et al., 2019). In Spain, the incidence of CP-Eco has evolved from isolated cases in 2013 (Oteo et al., 2015), to being detected in 10 different Spanish provinces in 2019 (Cañada-García et al., 2022).

Given the importance and the widespread dissemination of CP-Eco, the aim of this study is the phenotypic and genotypic characterization of a set of such isolates obtained from different Spanish regions over a period of five years.

2 Materials and methods

2.1 Study design and bacterial collection

As part of the ongoing Antibiotic Resistance Surveillance Program, the Spanish National Center for Microbiology (CNM) receives clinical carbapenem-resistant *E. coli* isolates for molecular characterization from Spanish hospitals. Participation in this program is voluntary and the criteria to send the isolates to the CNM is having a Minimum Inhibitory Concentration (MIC) to meropenem of over 0.12 mg/L (Giske et al., 2017).

Of the total CP-Eco isolates received in this Surveillance Program between January 2015 and December 2020, a subset was selected for further analysis according to the following criteria: i) one isolate for each type of carbapenemase per year was included from each of the collaborating hospitals, ii) in the event that there was more than one isolate for each carbapenemase type in the same year, the selection was prioritized based on the type of infection in which they were involved according to the following order: 1) invasive infections 2) other infections 3) colonizations; iii) in all cases, the first isolate received per year was selected. These selection criteria allowed for a temporal-spatial representative sample of the different types of carbapenemases circulating in *E. coli* in Spain. Not all hospitals had isolates from all the five years, and from some hospitals more than one isolate per year was included since they produced different carbapenemase types. The information sent along with the isolate from the participating hospitals included collection date, age and sex of the patients, sample type, whether the isolate was a colonizer or causing infections (as determined by the attending microbiologist), and isolate origin [hospital, healthcare center, or community setting; *i.e.* where the

infection is contracted without interaction with healthcare settings during the last 48 hours (Plachouras et al., 2018)].

2.2 DNA extraction and identification of carbapenemase genes

All the carbapenem-resistant *E. coli* isolates received at the CNM from 2015 to 2020 were cultured on MacConkey agar (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and incubated overnight at 37°C. DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR assays targeting the carbapenemase gene families *bla*_{OXA-48}, *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM} were performed (Ortega et al., 2016). All the isolates were stored at -80°C until used.

2.3 Antibiotic susceptibility testing

Antibiotic Susceptibility Testing (AST) was determined for the selected isolates with broth microdilution using the YDKMGN Sensititre™ Gram Negative panels (Thermo Fisher, Waltham, MA, USA) (International Organization for Standardization (ISO), 2006). E-tests using meropenem/vaborbactam and imipenem/relebactam and disc diffusion assays for cefiderocol (Liofilchem, Roseto degli Abruzzi, Italy) were performed and interpreted according to the EUCAST guidelines (European Society of Clinical Microbiology and Infectious Diseases (EUCAST), 2022). Additionally, E-tests using plazomicin (Liofilchem, Roseto degli Abruzzi, Italy) and fosfomycin (bioMérieux, Marcy l'Etoile, France) strips were performed and interpreted according to an FDA-approved breakpoint of susceptibility of ≤2mg/L for plazomicin (Cañada-García et al., 2022), and according to the EUCAST cutoff values for urinary tract infections (UTI) for fosfomycin due to lack of clinical breakpoints for other types of samples (European Society of Clinical Microbiology and Infectious Diseases (EUCAST), 2022), respectively.

Resistance to different antibiotic families was analyzed considering 14 categories: nine categories according to the classification proposed by Magiorakos et al. (Magiorakos et al., 2012) (extended spectrum cephalosporins, monobactams, carbapenems, aminoglycosides, fluoroquinolones, fosfonic acid derivatives, folic pathway inhibitors, glycolcyclines and polymyxins); and 5 additional categories: ceftazidime/avibactam (extended spectrum cephalosporin + diazabicycloctane inhibitor), imipenem/relebactam (carbapenem + diazabicycloctane inhibitor), meropenem/vaborbactam (carbapenem + boronic acid inhibitor), cefiderocol (siderophore cephalosporin) and plazomicin (extended spectrum aminoglycoside).

2.4 Whole-genome sequencing and read assembly

DNA was extracted from the selected isolates as described in section 2.2. Paired-end (2x150) libraries were then prepared using

the Nextera DNA Flex Library Preparation Kit and sequenced using Illumina NextSeq 550 (Illumina Inc., San Diego, CA, United States) according to the manufacturer's instructions. Quality of the reads was assessed using FASTQC (version 0.11.9), followed by *de novo* assembly using Unicycler (version 0.4.8) (Wick et al., 2017). The quality of the assembly was assessed using QUAST (version 5.2.0) and annotation was performed using Prokka (version 1.14.6) (Seemann, 2014). The obtained assemblies were deposited in the European Nucleotide Archive under the accession number PRJEB70795.

2.5 Phylogenetic analyses and diversity

Ridom SeqSphere+ (version 8.3.1; Ridom, Münster, Germany) was used to perform a core-genome Multi-Locus Sequence-Typing analysis (cgMLST) using a built-in scheme for *E. coli* containing 2,515 core genes, and to construct a minimum spanning tree based on allelic differences. ARIBA (version 2.6.2) (Hunt et al., 2017) was used to determine STs in accordance with the University of Warwick scheme. Diversity of the samples was calculated using a Simple Diversity Index [SDI (Gastmeier et al., 2006)]. Analysis of the *fimH* gene was done to subclassify ST131 isolates using the FimTyper tool [CGE server; <https://cge.cbs.dtu.dk>; (accessed 23/02/2024)].

2.6 Antibiotic resistance genes, virulence-associated genes, and plasmids

ARGs were analyzed by ARIBA (version 2.14.6) (Hunt et al., 2017) using the CARD database (<https://card.mcmaster.ca>; (accessed 23/02/2024)) and ResFinder (CGE server) with ID thresholds of 100% for β-lactamase variants and 98% for other resistance genes. In isolates resistant to cefiderocol, the sequences of the genes encoding PBP3 and the siderophore CirA, previously related to resistance to this antibiotic (Wang et al., 2022), were analyzed with ARIBA.

The VirulenceFinder tool (CGE server) (accessed 23/02/2024) was used to detect virulence-associated genes (five toxin-coding genes, four adhesin-coding genes, two siderophore-coding genes, one outer membrane protein-coding gene and one invasive protein-coding gene). PlasmidID [<https://github.com/BU-ISCIH/plasmidID>; (accessed 23/02/2024)] was used to map the reads against a curated plasmid database, perform *de novo* plasmid assemblies, and determine the presence of resistance and replicon genes (Pérez-Vazquez et al., 2019).

2.7 Statistical analyses

The differences in prevalence of the ARGs, virulence-related genes, STs, and sample characteristics were evaluated using the Fisher's exact test using GraphPad Prism (version 3.02; GraphPad Software, Inc., San Diego, CA, USA). *P* values of less than 0.05 were considered statistically significant.

2.8 Data availability

All the data generated for this study is available in the manuscript and its related [Supplementary Material](#). The sequenced raw reads have been deposited in the open-access European Nucleotide Archive database under the accession number PRJEB70795.

3 Results

3.1 Characteristics of all the CP-Eco isolates received from 2015 to 2020

From January 2015 until December 2020, a total of 593 CP-*E. coli* isolates were confirmed as carbapenems producers by the Spanish CNM Reference Lab after screening for MIC to meropenem of over 0.12 mg/L by the collaborating hospitals. [Supplementary Table S1](#) shows the characteristics and origin of the isolates, as well as the information received from the participating hospitals (total and per year). The isolates were received from 25 Spanish provinces and 59 hospitals. Two-hundred-ninety-nine (50.4%) of the isolates were from females and the average patient age was 77.8 years. Three-hundred-one (50.8%) isolates were colonizers, and the rest were causing infections (24 were unspecified). The most common infection was UTI (175; 29.5%), followed by wound/ulcer infections (32; 5.4%), bacteremia (25; 4.2%), and IAI (17; 2.9%). Four-hundred-forty-three (74.7%) were healthcare-associated cases, 391 from hospitals and 52 from other healthcare centers, and 126 (21.2%) were from community settings; 24 were unspecified.

Using real-time PCR carbapenemase genes were detected in all 593 isolates where *bla*_{OXA-48-like} was detected in 485 (81.8%), *bla*_{VIM-like} in 71 (12%), *bla*_{NDM-like} in 17 (2.9%), and *bla*_{KPC-like} in 17 (2.9%) isolates. Three isolates harbored two carbapenemases, one of each of the following combinations *bla*_{OXA-48} and *bla*_{KPC}, *bla*_{OXA-48} and *bla*_{NDM}, and *bla*_{VIM} and *bla*_{KPC}. In the community setting, the most frequent carbapenemase was *bla*_{OXA-48-family}, detected in 109 (86.5%) isolates. The relative frequency of these genes per year was maintained throughout the study period and there was no statistically relevant correlation between the types of carbapenemase gene, sample type, location of isolation, infection, colonization, and type of carbapenemase harbored by the isolates ($P > 0.05$).

3.2 Characteristics and population structure of the 90 selected CP-Eco isolates

Based on the criteria set in section 2.1, 90 isolates from 59 hospitals across 25 Spanish provinces were selected. [Supplementary Table S2](#) lists all the characteristics of these isolates. Sixty (66.6%) isolates were implicated in clinical infections, as determined by the doctor who treated the patient, and mainly were obtained from urine (27; 45%), blood (15; 25%) and abscesses and wounds (12;

20%). The remaining 30 (33.3%) isolates were considered colonizing and came mainly from rectal swabs and feces (26; 86.7%)

Forty-two (46.7%) of the isolates were obtained from the hospital setting, the majority of which harbored *bla*_{OXA-48} (24; 57.1%), followed by *bla*_{VIM-1} (8; 19%), *bla*_{NDM} (3 *bla*_{NDM-1} and 3 *bla*_{NDM-5}; 14.3% in total), *bla*_{KPC} (1 *bla*_{KPC-2} and 1 *bla*_{KPC-3}; 4.8% in total), *bla*_{OXA-162} (1; 2.4%), and both *bla*_{KPC-3} and *bla*_{OXA-48} (1; 2.4%). Twelve isolates were obtained from healthcare centers where 3 harbored *bla*_{OXA-48}, 3 *bla*_{VIM-1}, 2 *bla*_{NDM} (1 *bla*_{NDM-1} and 1 *bla*_{NDM-7}), 2 *bla*_{KPC} (1 *bla*_{KPC-2} and 1 *bla*_{KPC-3}), 1 (8.3%) *bla*_{OXA-162}, and 1 both *bla*_{KPC-3} and *bla*_{OXA-48}. From the community setting, 18 (20%) of the isolates were collected where 8 had *bla*_{OXA-48}, 4 *bla*_{VIM-1}, 3 *bla*_{NDM} (1 *bla*_{NDM-1} and 2 *bla*_{NDM-5}), and 3 *bla*_{KPC} (2 *bla*_{KPC-2} and 1 *bla*_{KPC-3}). The origin of 18 isolates could not be determined.

The carbapenemase genes detected among the infecting isolates were *bla*_{OXA-48} (30; 50%), *bla*_{VIM-1} (13; 21.7%), *bla*_{NDM} (5 *bla*_{NDM-1} and 3 *bla*_{NDM-5}; 13.3% in total), *bla*_{KPC} (3 *bla*_{KPC-2} and 3 *bla*_{KPC-3}; 10% in total), one *bla*_{OXA-162} (1; 1.7%), and both *bla*_{KPC} and *bla*_{OXA-48} (2; 3.3%). Among the colonizing isolates, the carbapenemases genes observed were *bla*_{OXA-48} (11; 36.7%), *bla*_{VIM-1} (8; 26.7%), *bla*_{NDM} (6; 20%) (3 *bla*_{NDM-5}, 2 *bla*_{NDM-1}, and 1 *bla*_{NDM-7}), *bla*_{KPC} (4; 13.3%) (3 *bla*_{KPC-2} and 1 *bla*_{KPC-3}), and *bla*_{OXA-162} (1; 3.3%). No statistically significant associations were found between the sample type, location of isolation, infection, colonization, and type of carbapenemase harbored by the isolates ($P > 0.05$).

MLST analyses showed that the 90 isolates belonged to 49 different STs (SDI=54.4) and only ST131 (14 isolates) and ST88 (6 isolates) were detected in more than five isolates. Population diversity was similar for isolates harboring *bla*_{OXA-48} (SDI=65.1) and *bla*_{NDM} (SDI=64.3), and was lower than those harboring *bla*_{KPC} (SDI=71.0) and *bla*_{VIM} (SDI=81.0). Ridom SeqSphere+ was used to construct a maximum-likelihood phylogenetic tree using the gene-by-gene approach and allelic distance from cgMLST. [Figure 1](#) shows a great genetic diversity of the CP-Eco studied alone and in relation to the types of carbapenemases they had. The average allelic distances in pairwise comparison of all the CP-Eco isolates was $2,120 \pm 555$, and only four main clusters with ≥ 4 isolates were identified. The first cluster included 14 (15.6%) CP-Eco isolates that belonged to ST131, had an average of 105 allelic differences (range:13-660), were collected from 7 provinces, and all 4 carbapenemase gene families were detected in them (including two with both *bla*_{OXA-48} and *bla*_{KPC-like}). The second-largest cluster was formed by 6 (6.7%) isolates belonging to ST88, had an average of 34 allelic differences (range:20-58), were collected from 5 provinces and 5 isolates harbored *bla*_{OXA-48}. Cluster 3 contained 4 (4.4%) isolates belonging to ST167 that all harbored *bla*_{NDM}, were collected from 3 provinces and had an average of 142 allelic differences (range:79-264). Cluster 4 contained 4 (4.4%) isolates pertaining to ST1193 collected from 2 provinces with an average allelic difference of 24 (range:20-32); two of them harbored *bla*_{OXA-48} and the other two *bla*_{NDM}. The rest of the isolates were grouped into STs with a frequency of 3 or less isolates. Applying a ≤ 5 allele differences threshold to identify possible transmission events, only one cluster of two OXA-48-producing ST127 isolates were detected.

Further typing of the *fimH* gene was conducted on the predominant ST131. Among the 14 ST131 isolates, 7 (50%) had

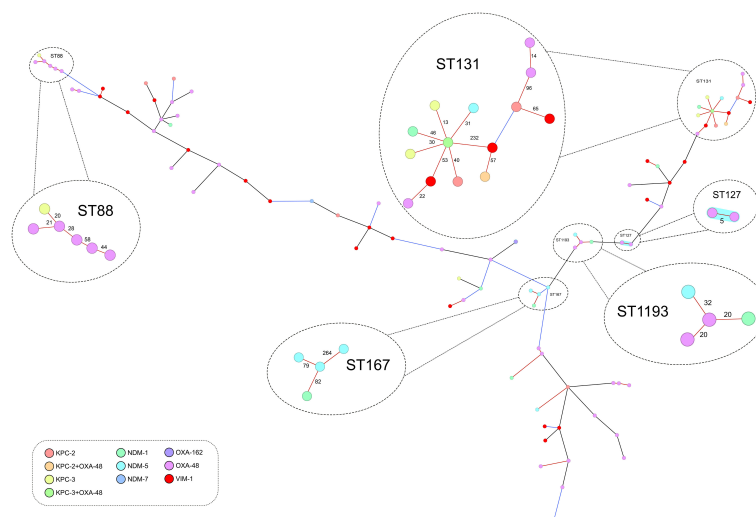


FIGURE 1

Minimum spanning tree showing the population structure of the 90 isolates analyzed through core-genome multi-locus sequence typing (scheme of 2,515 genes). The distance between the genes are not up to scale, black lines represent differences higher than 50% of the alleles considered in the scheme (>1257 alleles), blue lines differences between 10–50% (252 to 1257) and red lines differences lower than 10% (<252). Sequence types with four or more isolates are circled and amplified. The blue shading highlights the only group (containing two ST127 isolates) that have a genetic distance of ≤ 5 alleles (this group has also been amplified).

fimH30 indicative of belonging to clade C, 4 (28.6%) had *fimH41* (clade A), 1 (7.1%) had *fimH22* (clade B), 1 (7.1%) had *fimH298* (subtype of clade B), and 1 (7.1%) had *fimH1196* (no specific clade). *bla*_{CTX-M-15} and *bla*_{KPC} genes were more frequent in ST131 clade C isolates (71.4% and 57.1%, respectively) than in non-clade C isolates (14.3% and 28.6%, respectively) but these differences were not statistically significant ($P > 0.05$).

3.3 Characterization of the acquired antibiotic resistance genes

Table 1 shows the detected ARGs where an average of 5.6 ARGs were detected (range:1–13; excluding chromosomal genes and mutations). *bla*_{OXA-48-like} was detected in 43 (47.8%) isolates, *bla*_{VIM-1} in 21 (23.3%), *bla*_{NDM-like} in 14 (15.6%), *bla*_{KPC-like} in 10 (11.1%), and both *bla*_{OXA-48} and *bla*_{KPC-like} in 2 (2.2%) isolates. Isolates harboring *bla*_{VIM-1} (7.2; range:3–11) and *bla*_{NDM-like} (6.7; range:1–12) had more ARGs than those isolates harboring *bla*_{OXA-48} (5; range:1–13) and *bla*_{KPC-like} (4.6; range:1–12). Twelve isolates harbored only one ARG, six of them had *bla*_{OXA-48-like}, five had *bla*_{KPC-like}, and one had *bla*_{VIM-1}. Isolates belonging to ST88 had an average of 9.8 ARGs (range:7–13). *bla*_{OXA-48} was most frequently encountered in ST88 isolates (5; 11.6%), *bla*_{NDM-like} in ST167 (4; 28.6%), *bla*_{KPC-like} in ST131 (6; 54.6%) and *bla*_{VIM-like} in ST131 isolates (3; 14.3%). *bla*_{OXA-162} was detected in 2 isolates belonging to ST5968.

Acquired ESBL genes were detected in 27 (30%) isolates where *bla*_{CTX-M-15} was predominant ($n=13$; 48.1%). *bla*_{PER-2} was detected in a *bla*_{NDM-1}-harbouring isolate belonging to ST12. Regarding acquired AmpC genes, *bla*_{CMY} was detected in four isolates, three

of them belonged to ST167 and all four harbored *bla*_{NDM-like} (Supplementary Table S2).

The non- β -lactamase acquired ARGs present in at least 25% of the CP-Eco isolates were the sulfonamide-resistance-encoding *sul* ($n=64$, 71.1%; mainly *sul1*); trimethoprim-resistance-encoding *dfrA* ($n=56$, 62.2%; mainly *dfrA17*); tetracycline-resistance-encoding *tet* ($n=38$, 42.2%; mainly *tetA*); aminoglycoside-resistance-encoding *aac* (6') ($n=31$, 34.4%; mainly *aac*(6')-Ib) and *aadA* ($n=52$, 57.8%; mainly *aadA1*); chloramphenicol-resistance-encoding *cat* ($n=25$, 27.8%; mainly *catA1*); and quinolone-resistance-encoding *qnr* ($n=23$, 25.6%; mainly *qnrS1*) (Table 1; Supplementary Table S2). Twenty-five isolates had chromosomal mutations associated with resistance to fosfomycin and 2 additional isolates harbored the *fosA7.5* and *fosA3* acquired genes. These genes were located in chromosomal and plasmid contigs, respectively. The aminoglycoside methyltransferase gene *rmt* was detected in five isolates belonging to different STs, where 4 harbored *bla*_{NDM-1} and 1 *bla*_{NDM-5}.

3.4 Characterization of the plasmids carrying the carbapenem resistance genes

The median size of the detected plasmids was 63,598 bp with a median coverage of 98% and a median similarity of 100% with the respective entries in Genbank. IncL plasmids were the most frequently detected ($n=42$; 46.7%). Thirty-two of these harbored *bla*_{OXA-48}, eight harbored *bla*_{VIM-1}, and two harbored *bla*_{OXA-162}. The second most frequent plasmid type was IncFII detected in 12 (13.3%) isolates, eight of which harbored *bla*_{OXA-48} and four harbored *bla*_{NDM}. Five of the IncF-harboring isolates also belonged to clade C of ST131. IncN was detected in

TABLE 1 Genes and mutations associated with antibiotic resistance detected through whole-genome sequencing.

Carbapenemases	n (%)	ESBLs	n (%)	Other β -Lactamases	n (%)
<i>bla</i> _{OXA-48}	41 (45.6%)	<i>bla</i> _{CTX-M-15}	13 (14.4%)	<i>bla</i> _{OXA-1}	17 (18.9%)
<i>bla</i> _{VIM-1}	21 (23.3%)	<i>bla</i> _{CTX-M-14}	6 (6.7%)	<i>bla</i> _{CMY}	4 (4.4%)
<i>bla</i> _{NDM-1}	7 (7.8%)	<i>bla</i> _{SHV-12}	3 (3.3%)	<i>bla</i> _{TEM-30} & <i>bla</i> _{OXA-1}	1 (1.1%)
<i>bla</i> _{KPC-2}	6 (6.7%)	<i>bla</i> _{CTX-M-24}	2 (2.2%)	<i>bla</i> _{TEM-34}	1 (1.1%)
<i>bla</i> _{NDM-5}	6 (6.7%)	Others ²	3 (3.3%)	<i>bla</i> _{DHA-1}	1 (1.1%)
<i>bla</i> _{KPC-3}	4 (4.4%)				
<i>bla</i> _{OXA-162}	2 (2.2%)				
Others ¹	3 (3.3%)				
Chloramphenicol	n (%)	Fosfomycin	n (%)	Tetracycline	n (%)
<i>catA</i> (1&2) + <i>catB</i> (2&3)	11 (12.2%)	<i>ptsI</i> _V25I + <i>uhpT</i> _E350Q	14 (15.6%)	<i>tet</i> (A)	15 (16.7%)
<i>catB</i> (2&3)	8 (8.9%)	<i>uhpT</i> _E350Q	11 (12.2%)	<i>tet</i> (B)	12 (13.3%)
<i>cmlA1</i>	5 (5.6%)	Others ³	2 (2.2%)	<i>tet</i> (A) + <i>tet</i> (M)	1 (1.1%)
<i>floR</i>	4 (4.4%)				
<i>catA1</i>	3 (3.3%)				
<i>catA1</i> + <i>floR</i>	3 (3.3%)				
TMX	n (%)	Quinolones (chr.)	n (%)	Quinolones (acq.)	n (%)
<i>sul</i> (1,2&3)	43 (47.8%)	<i>gyrA</i> + <i>parC</i> + <i>parE</i>	37 (41.1%)	<i>qnrS</i> (1,2,&11)	15 (16.7%)
<i>dfrA</i> ⁴	29 (32.2%)	<i>gyrA</i> + <i>parC</i>	8 (8.9%)	<i>qnrB</i> (2,4&19)	5 (5.6%)
<i>dfrB1</i>	7 (7.8%)	<i>gyrA</i>	8 (8.9%)	Others ⁶	3 (3.3%)
<i>dfrA</i> (5&17) + <i>dfrB1</i>	2 (2.2%)	Others ⁵	3 (3.3%)		
AMEs	n (%)	Amino Methyl.	n (%)	Rifamycin	n (%)
<i>aad</i> (A1,A2&A16)	52 (57.8%)	<i>rmtC</i>	3 (3.3%)	<i>arr-3</i>	1 (1.1%)
<i>aac</i> (6')(-Ib&-Ib-cr)	30 (33.3%)	<i>rmtB1</i>	1 (1.1%)	<i>rpoB</i> _V146F	1 (1.1%)
<i>aac</i> (3)(-IIId,-IIe&-IVa)	13 (14.4%)	<i>rmtF1</i>	1 (1.1%)		
<i>ant</i> (2'')-Ia	3 (3.3%)				
<i>aph</i> (3')(-VI&-XV)	2 (2.2%)				

¹One of each: *bla*_{KPC-2} & *bla*_{OXA-48}, *bla*_{KPC-3} & *bla*_{OXA-48} and *bla*_{NDM-7}.

²One of each: *bla*_{CTX-M-27}, *bla*_{CTX-M-9} and *bla*_{PER-2}.

³One of each: *fosA3* and *fosA7.5*.

⁴Variants detected are 1, 5, 8, 12, 14, 17, 27 and 36.

⁵One of each: *parC*, *parE* and *gyrA* & *parE*.

⁶One of each: *qnrA1* & *qnrS2*, *qnrB1* & *qnrS1*, and *qnrVC1*.

"ESBL" stands for "Extended-Spectrum Beta-Lactamases", "Chr. mutations" refers to "chromosomal", "Acq." refers to "Acquired genes", "TMX" refers to Trimethoprim/Sulfamethoxazole, "Amino Methyl." refers to "16S rRNA Methylases that confer resistance to aminoglycosides", and "AMEs" refer to "Aminoglycoside Modifying Enzymes".

five (5.3%) isolates where four harbored *bla*_{VIM-1} and one harbored *bla*_{KPC-2}. IncP6 was detected in four isolates harboring *bla*_{KPC-2}, and IncFIB was detected in four of the five isolates harboring *bla*_{KPC-3}. Other plasmid types were detected with a frequency of two or less and it was not possible to fully reconstruct the carbapenemase-harboring plasmids in 20 isolates (Supplementary Table S2).

No other ARG was detected on the same plasmid harboring *bla*_{OXA-48-like} nor for those harboring *bla*_{KPC-like}, except for one isolate harboring *bla*_{KPC-2} that also harbored a class 1 integron with

the sequence *Int1-aac*(6')-*Ib-arr3-dfrA27-aadA16-qacEΔ1-sul1*. *bla*_{VIM-1} in IncL plasmids was found in a class 1 integron with the sequence *Int1-bla*_{VIM-1}-*aac*(6')-*Ib-dfrB1-aadA1-catB2-qacEΔ1-sul1*. In four other isolates, IncN plasmids harbored class 1 integron with *bla*_{VIM-1} but with a different combination of genes. Six isolates harbored *bla*_{NDM-5} (n=4), *bla*_{NDM-1} (n=1) or *bla*_{NDM-7} (n=1) in complex class 1 integrons with different combinations of aminoglycoside-resistance genes, including *rmtC*. Another isolate had *bla*_{NDM-1} and *rmtC* on the same plasmid, but they did not form part of an integron (Supplementary Table S2).

3.5 Antibiotic resistance profiles

Table 2 shows the AST results that were analyzed according to the categories described in section 2.3. All isolates had MICs > 0.12 mg/L to meropenem, harbored at least one gene of carbapenem resistance, and were resistant to at least one carbapenem (Table 2). The two isolates producing two carbapenemases presented relatively low MICs of 4 mg/L to

imipenem (both) and 4 and 1 mg/L to meropenem (each). Antibiotics with *in-vitro* activity against more than 90% of the isolates were colistin, plazomicin, ceftiderocol, and meropenem/vaborbactam. The *mcr* genes and known mutations in the PhoPQ and PmrAB encoding genes related to colistin resistance were not detected in the colistin-resistant isolate.

Forty isolates (44.4%) were resistant to more than six out of the 14 antibiotic categories considered. These isolates harbored *bla*_{VIM-1}

TABLE 2 Antibiotic susceptibility profiles of the 90 selected isolates determined through broth microdilution and interpreted according to the EUCAST guidelines.

Antibiotic Category	Antibiotic	Range S – R (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	S	SIE	R
Carbapenems	Ertapenem	0.25 – >2	2	>2	16 (17.8%)	N/A	74 (82.2%)
	Imipenem	≤0.5 – >16	2	16	53 (58.9%)	14 (15.6%)	23 (25.6%)
	Meropenem	≤0.12 – >16	1	16	68 (75.6%)	10 (11.1%)	12 (13.3%)
Carbapenem + Diazabicyclooctane	Imipenem + relebactam	≤2 – >2	1	8	61 (67.8%)	N/A	29 (32.2%)
Carbapenem + Boronate	Meropenem + vaborbactam	≤8 – >8	0.5	16	82 (91.1%)	N/A	8 (8.9%)
Extended Spectrum Cephalosporins	Ceftazidime	≤0.5 – >16	>16	>16	24 (26.7%)	13 (14.4%)	53 (58.9%)
	Cefotaxime	≤0.5 – >8	>8	>8	10 (11.1%)	13 (14.4%)	67 (74.4%)
Cefalosporin + Diazabicyclooctane	Ceftazidime + avibactam	≤0.5/4 – >16/4	1/4	>16/4	52 (57.8%)	N/A	38 (42.2%)
Cephalosporin + Siderophore	Cefiderocol	≤2 – >2	0.025	1	83 (92.2%)	N/A	7 (7.8%)
Monobactam	Aztreonam	≤0.5 – >32	≤0.5	>32	47 (52.2%)	5 (5.6%)	38 (42.2%)
Aminoglycosydes	Gentamicin	≤0.5 – >8	2	>8	51 (56.7%)	N/A	39 (43.3%)
	Amikacin	≤4 – >32	≤4	32	74 (82.2%)	N/A	16 (17.8%)
	Tobramycin	≤1 – >8	8	>8	44 (48.9%)	N/A	46 (51.1%)
Extended-Spectrum Aminoglycoside	Plazomicin	≤2 – >2	1.5	2	83 (92.2%)	N/A	7 (7.8%)
Folic Pathway Inhibitors	Trimethoprim + sulfamethoxazole	≤1/19 – >8/152	>8/152	>8/152	35 (38.9%)	3 (3.3%)	52 (57.8%)
Phosphonic Acid Derivative	Fosfomycin	≤8 – >8	2	4	82 (91.1%)	N/A	8 (8.9%)
Fluoroquinolones	Ciprofloxacin	≤0.06 – >2	>2	>2	27 (30.0%)	3 (3.3%)	60 (66.7%)
Glycylcyclins	Tigecyclin	≤0.25 – 4	0.5	1	71 (78.9%)	N/A	19 (21.1%)
Polimixins	Colistin	≤0.25 – >8	1	1	89 (98.9%)	N/A	1 (1.1%)

“MIC” stands for Minimum Inhibitory Concentration, “N/A” stands for “Not Applicable”, “R” stands for “Resistant”, “S” stands for “Susceptible”, and “SIE” stands for “Susceptible, Increased Exposure”.

(n=15; 37.5%), *bla*_{NDM-like} (n=13; 32.5%), *bla*_{OXA-48-like} (n=7; 17.5%), *bla*_{KPC-like} (n=4; 10%), and both *bla*_{KPC-3} and *bla*_{OXA-48} (n=1; 2.4%). Nine isolates (7 NDM-producers, 1 VIM-producer, and 1 OXA-48-producer) were only susceptible to three or four antibiotic categories. The most active agents against these isolates were colistin (9/9) and tigecycline (6/9).

Table 3 shows the antibiotic resistance rates of the CP-Eco isolates, grouped by the type of carbapenemase gene harbored. Isolates harboring *bla*_{OXA-48} had lower resistance rates towards meropenem and imipenem as compared to non-*bla*_{OXA-48}-harboring isolates (4.7% and 7% versus 21.3% and 46.2%, respectively; $P = 0.0003$). OXA-48 and ESBL co-producers (n=15) were significantly more resistant to ceftazidime (46.7%), cefotaxime (100%), and aztreonam (100%) than OXA-48 producers without ESBL (n=26, with 3.8%, 15.4%, and 3.8% resistance rates, respectively ($P < 0.05$)). No other β -lactamases combinations could be statistically studied in relation to antibiotic resistance profiles due to the very low number of isolates for each combination. All the isolates resistant to imipenem/relebactam (n=29), ceftazidime/avibactam (n=26) or meropenem/vaborbactam (n=8) were

metallo- β -lactamase-(MBL)-producers, except for one OXA-48-producer that was resistant to meropenem/vaborbactam and imipenem/relebactam, one OXA-48-producer resistant to imipenem/relebactam and ceftazidime/avibactam, and two isolates resistant to ceftazidime/avibactam producers of KPC-3/OXA-48 and OXA-48 carbapenemases, respectively. Three of these isolates presented insertions in the *ompC* gene in comparison with ertapenem-resistant isolates from this same study but susceptible to the rest of the carbapenems and to 3rd generation cephalosporins and their combinations. Insertions detected were E87_N88insD in one OXA-48 producer, and T225_A226insGLNGYG in one OXA-48- and one KPC-3/OXA-48-producing isolates. However, these insertions do not generate a premature stop codon. *ompF* mutations were not detected.

Six of the seven isolates resistant to cefiderocol were NDM-producers (4 *bla*_{NDM-5} and 2 *bla*_{NDM-1}) and one had *bla*_{VIM-1}. Four NDM-producers belonged to ST167 and had an insertion in the *pbp3* gene (Y334insYRIN), in addition to two other mutations in the same gene (E349K and I532L). Three of these isolates additionally had a frameshift mutation in the siderophore gene *cirA* (V89fs). Another

TABLE 3 Number and percentages of the CP-Eco isolates that are resistant to the tested antibiotics grouped by the carbapenemase gene that they harboured.

		<i>bla</i> _{OXA-48} (n = 43)	<i>bla</i> _{VIM} (n = 21)	<i>bla</i> _{NDM} (n = 14)	<i>bla</i> _{KPC} (n = 12)
Carbapenems	Ertapenem	41 (95.3%)	8 (38.1%)	14 (100.0%)	11 (91.7%)
	Imipenem	3 (7.0%)	5 (23.8%)	13 (92.9%)	2 (16.7%)
	Meropenem	2 (4.7%)	1 (4.8%)	8 (57.1%)	1 (8.3%)
Carbapenem + Diazabicyclooctane	Imipenem + relebactam	2 (4.7%)	13 (61.9%)	14 (100.0%)	0 (0.0%)
Carbapenem + Boronate	Meropenem + vaborbactam	1 (2.3%)	0 (0%)	7 (50.0%)	0 (0.0%)
Extended Spectrum Cephalosporins	Ceftazidime	21 (48.8%)	21 (100.0%)	14 (100.0%)	11 (91.7%)
	Cefotaxime	8 (18.6%)	21 (100.0%)	14 (100.0%)	10 (83.3%)
Cefalosporin + Diazabicyclooctane	Ceftazidime + avibactam	2 (4.7%)	21 (100.0%)	14 (100.0%)	1 (8.3%)
Cephalosporin + Siderophore	Cefiderocol	0 (0%)	1 (4.8%)	6 (42.9%)	0 (0.0%)
Monobactam	Aztreonam	13 (30.2%)	2 (9.5%)	11 (78.6%)	12 (100.0%)
Aminoglycosydes	Gentamicin	9 (21.0%)	18 (85.7%)	7 (50.0%)	4 (33.3%)
	Amikacin	3 (7.0%)	4 (19.0%)	7 (50.0%)	1 (8.3%)
	Tobramicin	12 (27.9%)	21 (100.0%)	8 (57.1%)	4 (33.3%)
Extended Spectrum Aminoglycoside	Plazomicin	0 (0.0%)	1 (4.8%)	5 (35.7%)	0 (0.0%)
Folic Pathway Inhibitors	Trimethoprim + sulfamethoxazole	20 (46.5%)	15 (71.4%)	11 (78.6%)	6 (50.0%)
Phosphonic Acid Derivative	Fosfomycin	3 (7.0%)	3 (14.3%)	2 (14.3%)	0 (0.0%)
Fluoroquinolones	Ciprofloxacin	26 (60.5%)	13 (61.9%)	13 (92.9%)	8 (66.7%)
Glycylcyclins	Tigecyclin	9 (20.9%)	4 (19.0%)	2 (14.3%)	4 (33.3%)
Polimixins	Colistin	1 (2.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

NDM-producer (ST131) had a truncation in *cirA* (W243trunc), and the final NDM-producer belonged to ST405, had the Y334insYRIN insertion in the *pbp3* gene, and a frameshift mutation in *cirA* (T508fs). The VIM-producer belonged to ST23 and had a frameshift mutation in *cirA* (T190fs). The median inhibition zone for NDM-producers was significantly lower compared to non-NDM-producers (20.5 versus 29.5; $P = 0.00001$).

There were six isolates that were resistant to all aminoglycosides, including plazomicin, and that carried *rmt* methyltransferases (3 *rmtC*, 1 *rmtF1* and 1 *rmtB1*), with five of them additionally having *bla*_{NDM-like}. All 16 amikacin-resistant isolates harbored *aac(6')-Ib* and/or methyltransferases genes, while the 46 tobramycin-resistant isolates had *aac(3)-II*, *aac(6')-Ib*, *ant(2'')-Ia* or *rmtB1* genes (Supplementary Table S2). Two NDM-producers were resistant to aztreonam with no other β -lactamase gene detected, while all other MBL-producers resistant to this antibiotic harbored ESBL and/or AmpC genes (Supplementary Table S2).

Finally, 82 isolates (91.1%) had fosfomycin MICs of ≤ 8 mg/L and were considered susceptible (using EUCAST criteria for UTIs). Of those, 21 isolates (25.6%) had chromosomal mutations associated with resistance to fosfomycin. Of the 8 isolates resistant to this antibiotic, two harbored *fosA* genes, three had the chromosomal mutations ptsI_V25I/uhpT_E350Q, one had the chromosomal mutation uhpT_E350Q, and in the last two no known genetic mutation or gene was identified. There was a significant correlation between harboring mutations or genes associated with fosfomycin resistance and phenotypic resistance to this antibiotic ($P = 0.003$).

3.6 Characterization of the virulence-associated genes

Table 4 shows the detected virulence-associated genes (13 virulence genes considered), and the number of infection-causing isolates harboring them. The isolates had a median of two virulence-associated genes (range=0-9) where adhesin-coding genes were the most prevalent (68.9%). Thirty-eight isolates (42.2%) had four or more virulence-associated genes, 19 of which were causing infections. The most common infection in this group was UTI (9), followed by bacteremia (5), IAI (2), wound infections (2), and abscess (1) (Supplementary Table S2). Isolates with 4 or more virulence-associated genes included three isolates belonging to ST131 and two belonging to ST88; and had similar averages of ARGs and susceptibility rates to the different antibiotic categories. Nineteen of these 34 isolates had *bla*_{OXA-48}, 8 had *bla*_{VIM-1}, 3 had *bla*_{NDM-5}, 2 had *bla*_{NDM-1}, 1 had *bla*_{KPC-3}, and 1 had both *bla*_{OXA-48} and *bla*_{KPC-3}. Of the isolates pertaining to ST131, all had *fimH* and over half of them harbored *pap* and *ompA*. Over half of those pertaining to ST88 harbored *fimH*, *iroN*, and *ompA*.

The adhesin-coding *pap* and the toxin-coding *sat* genes were found to be more frequent among isolates pertaining to ST131 ($P = 0.02$ and 0.0004 , respectively). Particularly, five of the 9 isolates positive for *pap* and five out of the six positive for *sat* belonged to clade C. Additionally, the adhesin-coding *fimH* gene had almost double the frequency among isolates harboring *bla*_{OXA-48} as compared to isolates harboring other carbapenemase genes ($P = 0.04$). There

were no statistically significant differences in the virulence-associated genes between bloodstream isolates and isolates from other sources.

4 Discussion

In this study, 90 representative CP-Eco isolates obtained from 25 Spanish provinces from 2015 to 2020 were characterized. The inclusion criteria were designed to achieve the greatest possible representation of geographic areas, clones, and carbapenemases, as well as to avoid overrepresentation due to outbreaks and/or endemics. They do not reflect the real prevalence of the carbapenemase genes; an approach to this more general view is provided in section 3.1, which mirrored the epidemiological situation of CP-Eco in Spain (Cañada-García et al., 2022). Notably, CP-Eco isolates were found in 25 Spanish provinces, and major STs, such as ST131, showed inter-provincial spread. The studied CP-Eco isolates were highly polyclonal, unlike other Enterobacterales, such as *Klebsiella pneumoniae* that tend to show less genetic diversity (Oteo et al., 2015; Lázaro-Perona et al., 2022).

The high-risk ST131 was the most prevalent in this collection; this ST also represented 36% of CP-Eco isolates from 16 countries between 2008 and 2013 (Patiño-Navarrete et al., 2020). In Spain, ST131 represented 16.5% of CP-Eco isolates collected between 2012-2014 (Ortega et al., 2016). Furthermore, isolates belonging to this ST harbored all the carbapenemase genes detected in this study, on different plasmids and in different combinations with other acquired ARGs. This demonstrates its potential to act as a vector for disseminating resistance genes in different scenarios. Half of the ST131 isolates caused infections, and all of them were community-acquired. Moreover, a significant association was found between ST131 and the virulence genes *sat* (toxin-coding) and *pap* (adhesin-coding), highlighting its potential to cause life-threatening community-acquired infections.

The ST167/NDM-5 clone, observed in this study, had been previously described (Poirel et al., 2022b) and had shown to spread rapidly across European borders (Linkevicius et al., 2023). These isolates were also resistant to cefiderocol and detected in three different provinces. Of note, 6/7 of the cefiderocol-resistant isolates were also NDM-producers, an association that has been previously drawn, especially among ST167 isolates having the same mutations in *pbp3* as shown in our study and a premature stop codon in the siderophore gene *cirA* (Wang et al., 2022). Additionally, NDM-producers were found to have tighter inhibition zones towards cefiderocol compared to non-NDM-producers, in line with recent findings where NDM-producers had elevated MICs towards this antibiotic (Poirel et al., 2022a). This hints at an interplay between NDM and cefiderocol resistance that warrants further investigation.

Another finding in our study is the detection of a relatively high rate of MBL-producers in isolates recovered from the community setting. The high average age of these patients could suggest an origin in nursing homes that are known reservoirs of CP-producing bacteria (Palacios-Baena et al., 2016) and whose flow of admissions-discharges from and to the community is difficult to trace. Moreover, *bla*_{VIM-1} was detected in small IncI plasmids similar to the highly transmissible ones that carried *bla*_{OXA-48} (Boyd et al.,

TABLE 4 Virulence-associated genes detected in the all 90 carbapenemase-producing *E. coli* isolates, in addition to the two most frequent sequence types and the number of isolates harboring these genes that were causing infections.

Adhesin-Coding Genes						
	All Isolates (n = 90)		ST131 Isolates (n = 14)		ST88 Isolates (n =6)	
Gene	n (%)	Causing Infection	n (%)	Causing Infection	n (%)	Causing Infection
<i>fimH</i>	65 (72.2%)	44	14 (100%)	9	4 (66.7%)	3
<i>pap</i>	30 (33.7%)	20	9 (62.3%)	7	2 (33.3%)	2
<i>sfa</i>	27 (30.3%)	18	6 (42.9%)	5	2 (33.3%)	2
<i>afa</i>	7 (7.9%)	6	0 (0%)	0	2 (33.3%)	2
Toxin-Coding Genes						
	All Isolates (n = 90)		ST131 Isolates (n = 14)		ST88 Isolates (n =6)	
Gene	n (%)	Causing Infection	n (%)	Causing Infection	n (%)	Causing Infection
<i>sat</i>	13 (14.6%)	7	6 (42.9%)	5	0	0
<i>vat</i>	11 (12.4%)	7	0 (0%)	0	0	0
<i>hlyA</i>	9 (10.1%)	7	2 (14.3%)	1	0	0
<i>cnf1</i>	9 (10.1%)	6	3 (21.4%)	1	0	0
<i>pic</i>	2 (2.2%)	2	0 (0%)	0	0	0
Siderophore-Coding Genes						
	All Isolates (n = 90)		ST131 Isolates (n = 14)		ST88 Isolates (n =6)	
Gene	n (%)	Causing Infection	n (%)	Causing Infection	n (%)	Causing Infection
<i>iroN</i>	15 (16.9%)	14	0 (0%)	0	4 (66.7%)	4
<i>iutA</i>	1 (1.1%)	0	0 (0%)	0	0 (0%)	0
Outer Membrane Protein-Coding Genes						
	All Isolates (n = 90)		ST131 Isolates (n = 14)		ST88 Isolates (n =6)	
Gene	n (%)	Causing Infection	n (%)	Causing Infection	n (%)	Causing Infection
<i>ompA</i>	69 (77.5%)	47	10 (71.4%)	7	4 (66.7%)	4
Invasive Protein-Coding Genes						
	All Isolates (n = 90)		ST131 Isolates (n = 14)		ST88 Isolates (n =6)	
Gene	n (%)	Causing Infection	n (%)	Causing Infection	n (%)	Causing Infection
<i>ibeA</i>	4 (4.5%)	2	1 (7.1%)	0	0 (0%)	0

2022). This combination was detected in isolates belonging to different STs, as previously described in *K. pneumoniae* (Cañada-García et al., 2023), and was predominantly found in healthcare-associated settings. This raises concern regarding the fast dissemination of *bla*_{VIM-1}, especially in Spain where the prevalence of VIM-producers is higher than other countries (Johnston et al., 2021; Cañada-García et al., 2022). Of note, *bla*_{OXA-48} was in IncFII plasmids in eight isolates, an observation that was reported in another study though *bla*_{OXA-48} was part of a different transposon (Moussa et al., 2020).

The CP-Eco isolates harboring *bla*_{OXA-48} were significantly more susceptible to imipenem and meropenem compared to CP-Eco isolates harboring other carbapenemases, in accordance with the known weaker hydrolytic activity of this enzyme towards

carbapenems (López-Camacho et al., 2019). Moreover, all OXA-48-like-producers and all but one KPC-producer did not harbor any other ARG on their plasmids, unlike other Enterobacterales such as *K. pneumoniae* where it is typical to find other ARGs with *bla*_{KPC} (Cañada-García et al., 2022; Cañada-García et al., 2023). In contrast, 30-40% of MBL-producers were resistant to more than six antibiotic categories as defined in this study. All the “new” antibiotics and antibiotic/inhibitor combinations, except imipenem/relebactam, showed over 75% *in-vitro* activity towards the CP-Eco isolates. Resistance to imipenem/relebactam, meropenem/vaborbactam or ceftazidime/avibactam was mainly due to MBL production (Galani et al., 2019). Three of the only four isolates resistant to at least one of these combinations had insertions in the OmpC porin gene, in comparison with sequences

from OXA-48-producing isolates of this study only resistant to ertapenem but susceptible to the rest of the carbapenems and 3rd generation cephalosporins. The presence of mutations in the main porins of *Enterobacterales* has previously been related to resistance to the new beta-lactamase inhibitors combinations (Navarro-Carrera et al., 2022). VIM-producers appeared to be more resistant towards imipenem/relebactam than imipenem alone, which has been previously reported in another study (Hernández-García et al., 2022). However, this was mainly caused by the EUCAST cutoff values (European Society of Clinical Microbiology and Infectious Diseases (EUCAST), 2022) used to determine susceptibility since six of the eight VIM-1 producing isolates resistant to imipenem/relebactam but susceptible to imipenem had MICs of 4 mg/L for both antibiotics. There were also seven NDM-producers that were susceptible to meropenem/vaborbactam according to the EUCAST guidelines. However, these isolates had elevated MICs, and most would have been considered resistant in case the CLSI guidelines were followed (Jean et al., 2019).

Six isolates were resistant to plazomicin, five of them harbored *bla*_{NDM-like} and were the only isolates in which *rmt* methyltransferases were detected. Furthermore, unlike other *Enterobacterales* in which *rmt* methyltransferase gene seems to disseminate via clonal expansion (Arca-Suárez et al., 2022), in our collection this gene was detected in isolates from different STs and were present in different plasmids.

Fosfomycin is one of the main antibiotics used to treat community-acquired uncomplicated cystitis caused by *E. coli* in Spain (Sojo-Dorado et al., 2022). In this study, most of the isolates that had mutations associated with resistance to this antibiotic harbored chromosomal mutations, and only two isolates had *fos* genes, similarly to what was previously described (Falagas et al., 2019). Moreover, the *fosA7* genes detected in the chromosome of one of the isolates has also been described to be able to be present there (Pan et al., 2021). The correlation between resistance to fosfomycin and the presence of resistance mechanisms against this antibiotic must be taken with caution since cut-off points for UTI have been used (the only ones established by EUCAST), of which there were only 26 isolates in this collection. Moreover, although a good agreement was reported between using E-test strips and disk diffusion assays (Hirsch et al., 2015; Goer et al., 2022), the EUCAST does not explicitly recommend the use of E-tests for the determination of cutoff values for fosfomycin. Nevertheless, these isolates could be displaying a heteroresistant phenotype that appeared to be susceptible in our testing methods and criteria (Campos et al., 2020).

In conclusion, our data shows a wide range of genetically diverse CP-*E. coli* isolates present across several Spanish geographical areas, with a heterogeneous set of ARGs, plasmids, and virulence-associated genes. These findings highlight the potential of this bacteria to acquire and disseminate ARGs, and to cause life-threatening, difficult-to-treat infections in different settings, including the community. The spread of CP-Eco in the community, like what already happened with CTX-M-15-producing *E. coli*, would pose a threat of great impact to the general health of the population. The integration of WGS in the

surveillance and diagnosis of CP-Eco, in line with ECDC recommendations, can help control and minimize this impact.

Data availability statement

All the data generated for this study is available in the manuscript and its related [Supplementary Material](#). The sequenced raw reads have been deposited in the open-access European Nucleotide Archive database under the accession number PRJEB70795.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1390966/full#supplementary-material>

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A novel pathogenic species of genus *Stenotrophomonas*: *Stenotrophomonas pigmentata* sp. nov

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Introduction: *Stenotrophomonas* is a prominent genus owing to its dual nature. Species of this genus have many applications in industry and agriculture as plant growth-promoting rhizobacteria and microbial biological control agents, whereas species such as *Stenotrophomonas maltophilia* are considered one of the leading gram-negative multi-drug-resistant bacterial pathogens because of their high contribution to the increase in crude mortality and significant clinical challenge. Pathogenic *Stenotrophomonas* species and most clinical isolates belong to the *Stenotrophomonas maltophilia* complex (SMc). However, a strain highly homologous to *S. terrae* was isolated from a patient with pulmonary tuberculosis (TB), which aroused our interest, as *S. terrae* belongs to a relatively distant clade from SMc and there have been no human association reports.

Methods: The pathogenicity, immunological and biochemical characteristics of 610A2^T were systematically evaluated.

Results: 610A2^T is a new species of genus *Stenotrophomonas*, which is named as *Stenotrophomonas pigmentata* sp. nov. for its obvious brown water-soluble pigment. 610A2^T is pathogenic and caused significant weight loss, pulmonary congestion, and blood transmission in mice because it has multiple virulence factors, haemolysis, and strong biofilm formation abilities. In addition, the cytokine response induced by this strain was similar to that observed in patients with TB, and the strain was resistant to half of the anti-TB drugs.

Conclusions: The pathogenicity of 610A2^T may not be weaker than that of *S. maltophilia*. Its isolation extended the opportunistic pathogenic species to all 3 major clades of the genus *Stenotrophomonas*, indicating that the clinical importance of species of *Stenotrophomonas* other than *S. maltophilia* and potential risks to biological safety associated with the use of *Stenotrophomonas* require more attention.

KEYWORDS

Stenotrophomonas, tuberculosis, pathogen, multiple-drug resistance, pigment

1 Introduction

Stenotrophomonas is a member of the family *Xanthomonadaceae*. Because of advancements in molecular biology and identification technology, the number of species in this genus has increased rapidly in recent years. Based on our interpretation of the current available NCBI taxonomy, from the first species, *Stenotrophomonas maltophilia*, isolated in 1885 to 2011, 16 strains have been isolated within 117 years, and 12 species were identified within 9 years from 2016 to 2024. *Stenotrophomonas* is ubiquitous and associated with a wide range of habitats, including humans, animals, plant hosts, and extreme environments. Most members of *Stenotrophomonas* are particularly known for producing protective osmotic substances and are considered as plant rhizosphere growth-promoting bacteria. They also have valuable applications in the fields of agriculture and industry as emerging sources of biodegradation and substitutes for synthetic fungicides (Kumar et al., 2023). *Stenotrophomonas* strains can interact with other microorganisms on plant surfaces and in the soil through biofilms, inhibit plant pathogenic fungi and viruses, and alter the composition of rhizosphere microorganisms through extracellular enzyme decomposition and competition for iron. By inducing the enrichment of plant hormones, such as jasmonic acid, and upregulating the transcription level of jasmonic acid-sensitive genes, *Stenotrophomonas* strains help plants defend against various crop pests, such as *Spodoptera litura*. Their function in promoting plant growth is through the production of auxin and hydrogen cyanide; dissolution of phosphorus and potassium salts; nitrogen fixation; enzymatic degradation of soil odour, explosive pollutants, keratin, macrocyclic hydrocarbons, nitrophenols, and other substances; removal of various chemical pesticides, insecticides, and environmental pollutants; and effective bioremediation of agricultural soil without harming the ecosystem (Kumar et al., 2023).

A few species of this genus are pathogenic to humans and are represented by *S. maltophilia*. As a globally emerging organism, *S. maltophilia* is recognised for its multi-drug resistance (Gil-Gil et al., 2020), ability to cause various infections in the human body, and high contribution to the increase in crude mortality (Tan et al., 2014). Based on global clinical data, the attributed mortality rates for pulmonary infection and bacteraemia caused by this bacterium are as high as 30% and 65%, respectively (Zhou et al., 2013). *S. maltophilia* is listed by the World Health Organization as one of the leading gram-negative, multi-drug-resistant bacterial pathogens in hospitals (Gröschel et al., 2020) and poses a great clinical challenge (Mojica et al., 2022; Tamma et al., 2022).

Because of their phenotypic and genotypic diversity, the taxonomic status of the genus *Stenotrophomonas* and *S. maltophilia* has changed several times. Besides the species in the *Stenotrophomonas maltophilia* complex (SMc), *S. maltophilia* (Chang et al., 2015; Patil et al., 2018; Bansal et al., 2021; Brooke, 2021) and *S. seipia* (Gautam et al., 2021), only *S. pavanii* (Iebba et al., 2018), *S. acidaminiphila* (Ježek et al., 2020; Zhang et al., 2022)

and *S. rhizophila* (Chao et al., 2022) have been isolated from human samples. The clinically important species *S. africana* (Coenye et al., 2004) is classified as a synonym of *S. maltophilia*, whereas *S. pavanii* has been removed from SMc (Rouf et al., 2011; Patil et al., 2018; Maturana and Cardenas, 2021; Deng et al., 2022). Most of the clinical isolates belong to the 23 monophyletic lineages of SMc (Gröschel et al., 2020), and only *S. acidaminiphila* and *S. rhizophila* have no close genetic relationship to *S. maltophilia*.

In our previous small surveillance conducted in a district tuberculosis (TB) hospital in Beijing, we found that *Stenotrophomonas* was a major co-occurring species of *Mycobacterium tuberculosis*, with a crude isolation rate of 6.74%. *Stenotrophomonas* may be an important opportunistic pathogen for patients with TB, similar to that for patients with cystic fibrosis (Li et al., 2023). On the basis of 16S rDNA sequencing, 2 out of 9 *Stenotrophomonas* isolates did not belong to *S. maltophilia*, and one had the highest identity with *S. terrae* strain AFS037341 (99.24%) and *S. humi* strain AFS068096 (98.96%). These similarity values were more than 98.65%, which has been previously used as the threshold for differentiating *Stenotrophomonas* species (Deng et al., 2022). To the best of our knowledge, *S. terrae* and its closely related species have not been isolated from human resources, which sparked our interest in the pathogenicity of these strains. However, after PacBio whole-genome sequencing, the average nucleotide identity (ANI) between the strain 610A2^T, which was highest similar to *S. terrae*, and all existing species of the genus *Stenotrophomonas* was less than 88%. Therefore, in addition to animal experiments and pathogenicity assessments, we conducted a systematic evaluation of 610A2^T according to the description required for new taxa.

2 Materials and methods

2.1 Sources of isolates

Strain 610A2^T was isolated from a 26-year-old male patient with pulmonary TB under treatment at the tuberculosis clinic of the Chaoyang District Center for Disease Control and Prevention (39.89° N, 116.40° E) in our previous surveillance under ethics approval No. ICDC-2022010 (Li et al., 2023). It was first identified as *Stenotrophomonas* by amplification and sequencing with 16S rDNA universal primers for bacteria (16S-U: 5'AGA GTT TGA TCM TGG CTC AG 3' and/L: 5' CCG TCA ATT CMT TTR AGT TT 3').

2.2 Whole-genome sequencing and phylogenetic analysis

Genome of 610A2^T was sequenced using the PacBio sequel II and DNBSEQ platform at the Beijing Genomics Institute (BGI, Shenzhen, China). Four SMRT cell zero-mode waveguide arrays for sequencing were used to generate the sub-read set. The PacBio subreads (length < 1 kb) were removed. Canu and GATK (<https://www.broadinstitute.org/gatk/>) were used for self-correction and

single-base corrections, to improve the accuracy of the genome sequences.

Gene prediction was performed using glimmer3 with Hidden Markov models (<http://www.cbcu.umd.edu/software/glimmer/>). Other genomic elements, such as RNA, tandem repeats, genomic regions, small satellite DNA, and microsatellite DNA, were identified using tRNAscan SE, RNAmmer, Tandem Repeat Finder (<http://tandem.bu.edu/trf/trf.html>), Genomic Island Suite of Tools (<http://www5.esu.edu/cpsc/bioinfo/software/GIST/>), PHAge Search Tool (PHAST, <http://phast.wishartlab.com>), and CRISPRFinder. Eleven databases, namely, KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.kegg.jp>), COG (Clusters of Orthologous Groups, <http://www.ncbi.nlm.nih.gov>), NR (Non-Redundant Protein Database databases, <ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz>), Swiss-Prot (<http://ngdc.cnc.ac.cn>), GO (Gene Ontology, <http://www.geneontology.org>), TrEMBL, EggNOG (<http://eggnog.embl.de>), VFDB (Virulence Factors of Pathogenic Bacteria, <http://www.mgc.ac.cn>), ARDB (Antibiotic Resistance Genes Database, <http://www.argodb.org/>), CAZy (Carbohydrate-Active enZymes Database, <http://www.cazy.org>), and T3SS (Type III secretion system effector protein, EggNOG database 1.0.1, <http://effectivedb.org>), were used for general function annotation and pathogenicity/drug resistance analysis.

Complete 16S rDNA gene sequences were extracted from the sequenced genome and reference genomes of 26 species of the genus *Stenotrophomonas*, except for *S. detusculanense* and *S. indologenes*, which do not have publicly available genome sequences. ClustalX was used for multiple sequence alignments, and a bootstrap neighbour-joining (NJ) phylogenetic tree was constructed using Treeview (1.6.6) with 1000 replicas. ANI was estimated using the ANI calculator (<http://enve-omics.ce.gatech.edu/ani/>). Synteny was performed using MUMmer and BLAST. Core/Pan genes were clustered by CD-HIT rapid clustering of similar proteins software with a threshold of 50% pairwise identity and 0.7 length difference cutoff in amino acid. Hcluster_sg software was used to perform gene family clustering, and a phylogenetic tree was constructed using TreeBeST with the NJ method.

2.3 Examination of phenotypic and biochemical characteristics

The strain's growth ability was tested on 4 media, tryptone soy (TSA; Difco), Luria-Bertani (LB; Difco), Columbia blood agar (OXOID), and Mueller-Hinton (MH; Difco), at 37 °C for 24–72 h. Growth at different temperatures (4, 15, 20, 25, 28, 35, 37, 40, and 45 °C), pH (pH 4–11, at intervals of 1 pH unit, 37 °C) and NaCl concentrations (0–10%, w/v, in intervals of 1%) was also evaluated using LB broth and agar at 37 °C. Cellular motility was monitored using 0.75% semi solid agar medium, and morphological features were observed using a transmission electron microscope (HT7700; Hitachi, Japan). Utilisation of carbon sources and enzyme production were tested with API 20NE (48 h, 28 °C), API ZYM (4 h, 28 °C), and API 50 CH (inoculated with AUX medium, 48 h, 28 °C) (20050, 50300, 25200; BioMerier France, France), according to the manufacturer's instructions. Cellular fatty acids and matrix-

assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) were analysed using the Sherlock Microbial Identification System (MIDI) with the MIDI Sherlock software program (version 6.3), RTSBA6 (6.21) library, and Autoflex speed TOF/TOF (Bruker Daltonics GmbH, Germany), according to the manufacturers' instructions.

2.4 Antibiotic susceptibility testing

Two kinds of AST plates, the customised AST plate for Chinese Pathogen Identification Net (CHNENF, Trek Diagnostic Systems Ltd, West Sussex, United Kingdom) and the SensititreTM MYCOTB minimum inhibitory concentration (MIC) plate (Trek Diagnostic Systems, Cleveland, OH, USA), were used to determine the strain's sensitivity to 17 drugs commonly used for gram-negative strains and 12 anti-TB drugs. MICs were determined according to the standards of the Clinical and Laboratory Standard Institute (CLSI, 2018) and manufacturer's instructions.

2.5 Biofilm formation

Biofilm formation was determined using crystal violet staining. Fresh bacterial suspension was prepared and adjusted to 0.5 McFarland. After 1:30-fold dilution with tryptone soy broth (TSB; Difco), 150 µL/well suspension was added to a 96-well flat-bottom microtitre plate, and the plate was incubated at 37 °C. At each time point, bacterial turbidity (OD_{630 nm}) was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad 680; Bio-Rad Laboratories, Japan). The cultures were discarded, and the wells were washed 3 times with PBS (pH 7.3) to remove planktonic cells. The biofilms were stained with 200 µL/well of 0.1% crystal violet for 15 min. The wells were washed again, and 200 µL of 99% ethanol was added and OD_{403 nm} was measured after 15 min. The biofilm index was calculated as OD_{403 nm}/OD_{630 nm}. Six clinically isolated *S. maltophilia* strains, including strain 11066 (GDMCC 1.4335), were used as controls.

2.6 Animal infection experiment

Animal experiments were conducted with the approval of the Laboratory Animal Welfare & Ethics Committee of the National Institute for Communicable Disease Control and Prevention (Issue number 2023-021).

Using a random number method, female Kunming mice aged 6–7 weeks and weighing 30–35 g were randomly divided into infection and control groups. Five mice in each infection group were intranasally inoculated with 50 µL of 1.5×10^8 CFU/mL of tested strains, and mice in the control groups were inoculated with PBS. At 4 h, 12 h, 1 day, 2 days, 3 days, 5 days, and 7 days post-infection, the mice were sacrificed and the lung and spleen tissues were collected aseptically.

The left lobes of the lungs were fixed in 10% neutral formalin for 24 h, followed by tissue processing and paraffin embedding. The

paraffin blocks were sectioned at 2 μ m, stained with haematoxylin and eosin, and blindly examined under a microscope by an expert in the field of laboratory animal pathology. Pulmonary clearance and occurrence of disseminated infection were monitored via quantitative bacteriology of the lung and spleen homogenates, respectively. Briefly, tissues were homogenised (1000 rpm) on ice in 1 mL of sterile normal saline by using a homogeniser (RZ-GR96A; Beijing Guoke Rongzhi Biotechnology Co., Ltd., China). Then, 100 μ L of homogenates and 10-fold serial dilutions were inoculated on LB agar. The number of colonies was counted 24–48 h after incubation at 37°C. Bacterial colony counts were normalised according to the wet tissue weight and calculated as CFU/g. The levels of murine tumour necrosis factor alpha (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN- γ), interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, and IL-17A were quantified using Luminex technology (R&D Systems, Minneapolis, MN, USA) and reagents (12002798; BIO-RAD, USA).

2.7 Statistical analysis

For comparison of multiple groups, one-way ANOVA was performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA).

3 Results

3.1 Genomic characteristics and classification into the genus *Stenotrophomonas*

The total length of 610A2^T complete genome was 4,681,496 bp, and GC content was 63.29% with an N50 length of 11,086 bp. A total of 4,070 CDSs, 70 tRNAs, 6 5S rRNAs, 4 16S rRNAs, 4 23S rRNAs, 37 sRNAs, 353 tandem repeats, 175 minisatellite DNAs, and 90 microsatellite DNAs were annotated.

In the phylogenetic tree based on the complete 16S rDNA gene (1545 bp), 610A2^T was clustered in a clade including *S. terrae*, *S. nitritireducens*, *S. humi*, and *S. pictorum* and formed a subclade between *S. terrae* and *S. humi* (Figure 1). Dispensable gene heat maps and phylogenetic trees based on corePan and genefamily1 results showed completely consistent positioning (Figures 2A, B). When compared with the 26 *Stenotrophomonas* type species, 610A2^T displayed lower ANI values ranging from 87.53 to 80.22, less than 95%, the generally accepted threshold for species (Maturana and Cardenas, 2021; Deng et al., 2022). Synteny analyses at the nucleotide and amino acid levels were compared with 8 type strains: *S. humi* DSM 18929, *S. seipilia* SM16975, *S. geniculata* FLMAT1, *S. maltophilia* NCTC10257, *S. terrae* DSM 18941, *S. nitritireducens* DSM 12575, *S. rhizophila* DSM 14405, and *S. acidaminiphila* T0-18.

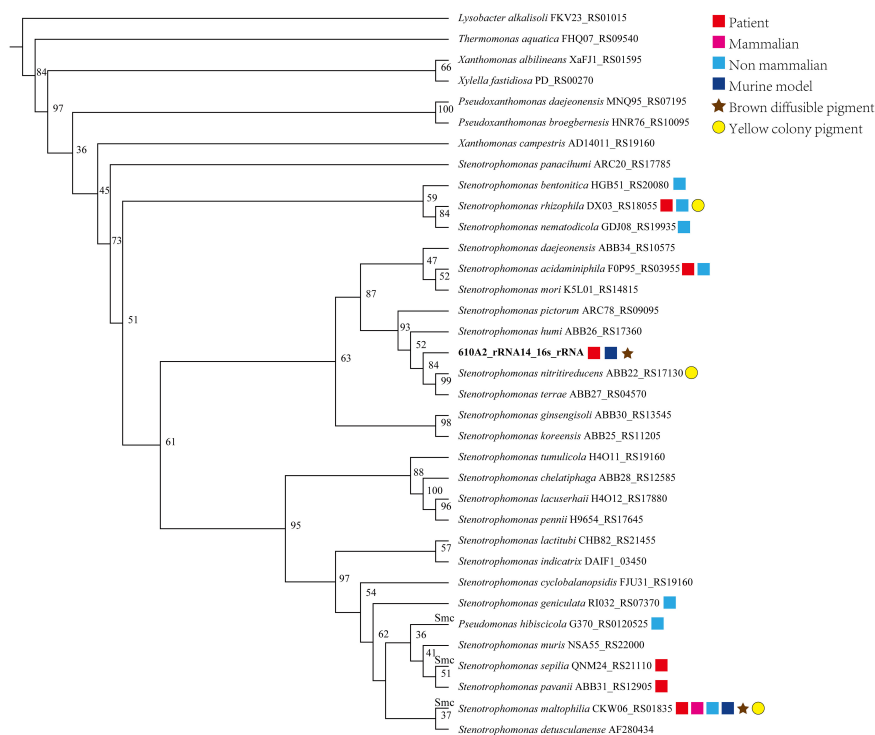


FIGURE 1

Phylogenetic tree created on the basis of complete 16S rDNA gene sequences of 610A2^T and reference genomes of 26 species *Stenotrophomonas*. ClustalX was used for multiple sequence alignments, and a bootstrap neighbour-joining (NJ) phylogenetic tree was constructed using Treeview (1.6.6) with 1000 replicas. Species isolated from patients or animals or tested on a murine model are marked with symbols following their species name and 16S rDNA locus_tag.

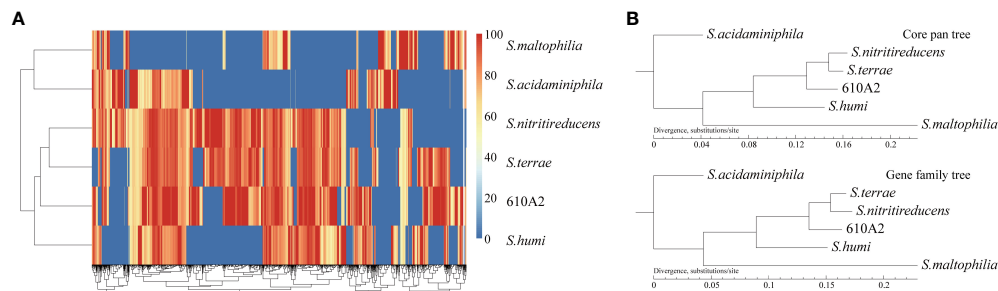


FIGURE 2
Gene heat map (A) and phylogenetic trees based on corePan and genefamily1 results (B).

S. terrae had the highest homology, with a gene median identity of 91.34% and map length rate of 63.327% (Table 1). These data suggest that 610A2^T represents a novel species within the genus *Stenotrophomonas*, and it is closely related to, but distinct from, *S. terrae*. The name *Stenotrophomonas pigmentata* sp. nov. is proposed for the distinct brown water-soluble pigment it produces.

3.2 Pathogenicity in animal infection experiment

At the infection dose of 50 μ L of 1.5×10^8 CFU/mL, no mortality was observed in either the infection or control group. The mice infected with 610A2^T showed a weight loss of $3.9 \pm 0.9\%$, $8.0 \pm 0.7\%$, $6.4 \pm 1.3\%$, $2.3 \pm 1.2\%$, $0.8 \pm 1.3\%$ at 12 h, 1 day, 2 days, 3 days, and 5 days post-infection, respectively. On the seventh day, weight gain resumed. In addition, no significant differences were observed in the physical signs between the infection and control groups.

The bacterial load per tissue weight (g) in the lung measured via viable count was $1.1 \pm 1.5 \times 10^7$ CFU/mL-g at 4 h, $1.8 \pm 3.6 \times 10^5$ CFU/mL-g at 12 h, and $2.0 \pm 4.0 \times 10^3$ CFU/mL-g at 1 day. Bacteria could not be isolated from lung homogenates 2 days post-infection.

610A2^T was isolated only from the spleen homogenate of all infected mice within 4 h, with a viable bacterial count of $1.1 \pm 2.2 \times 10^3$ CFU/mL g, indicating that 610A2^T has strong invasive ability but the host can also quickly clear it.

Histological examination showed that pulmonary damage in the infected group constantly included alveolar septa thickening, congestion, bleeding, inflammatory cell infiltration, compensatory alveolar dilation, mucosal epithelial oedema, and vacuolar degeneration (Figure 3). Although the viable count showed that the bacteria in the lungs had been cleared after 2 days, obvious bloody exudate and a large number of inflammatory cells were detected in the alveoli in 1–3 days, which is consistent with the symptoms for pulmonary congestion. After 7 days, the lung structure of the infected group was restored to normal, whereas that of the control group was normal.

Eight of the 9 quantified cytokines showed significant differences between infected and control groups (Figure 4). The means of TNF- α , GM-CSF, IL-4, IL-6, IL-10, and IL-17A at each timepoint were higher than those of the control group, but only the values at 4 h had statistical significance because of the variability caused by individual differences. IFN- γ and IL-2 showed a consistent trend of change, lagging behind other cytokines and showing significant differences from the control group on the 5th

TABLE 1 ANI values and synteny analyses at the nucleotide and amino acid levels between 610A2 and 8 type strains.

Species/ Strain name	ANI (%)	Nucleotide levels			Amino acid levels					
		Map Num	Map Length	Rate (%)	Aligned	Target Percent (%)	Query Genes	Query Percent (%)	Identity Mean	Identity Median
<i>S. humi</i>	83.94	459	1033761	22.082	2965	72.85	3612	82.09	85.14	88.01
<i>S. sepiolia</i>	81.05	188	265466	5.671	2535	62.29	4158	60.97	74.76	76.92
<i>S. geniculata</i>	81.26	196	302519	6.462	2570	63.14	4822	53.3	74.93	77.17
<i>S. maltophilia</i>	81.16	184	275081	5.876	2582	63.44	4007	64.44	74.93	77.155
<i>S. terrae</i>	87.53	2630	2964645	63.327	3201	78.65	3770	84.91	91.34	94.26
<i>S. nitritireducens</i>	87.47	2112	2787879	59.551	3126	76.81	3812	82	91.13	94.04
<i>S. rhizophila</i>	81.18	204	289657	6.187	2614	64.23	3938	66.38	75.46	77.9
<i>S. acidaminiphila</i>	83.08	343	686404	14.662	2598	63.83	3617	71.83	81.13	83.62

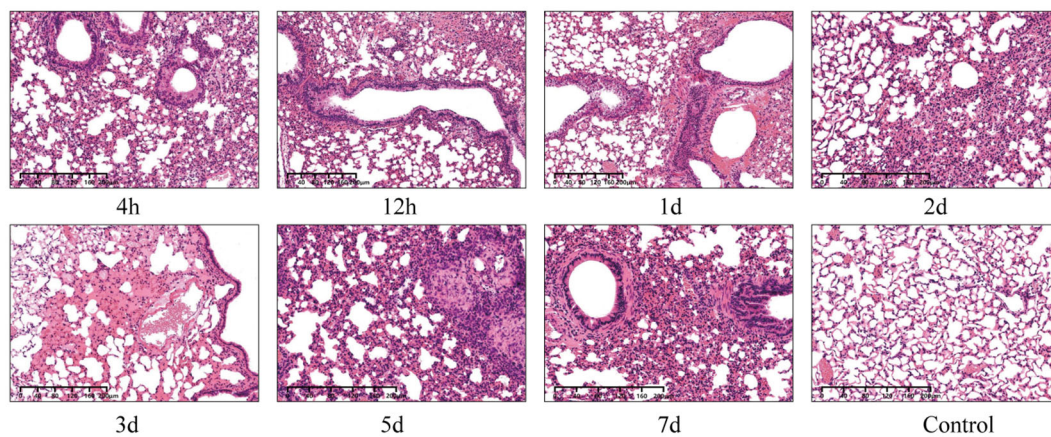


FIGURE 3

Microscopic images of lung histopathology of KM mice intranasally infected with 610A2^T at 7 timepoints (4 h, 12 h, 1 day, 2 days, 3 days, 5 days, and 7 days post-infection). The lung structure of the control group was normal, whereas infiltration of inflammatory cells, compensatory alveolar dilation, mucosal epithelial oedema, and even obvious bloody exudate was observed in the infected lung sections.

day; however, at this time point, TNF- α and IL-6 had recovered to levels no different from the control group.

3.3 Prediction of virulence factors

Functional annotation revealed that the 610A2^T genome contained 304 virulence factors of pathogenic bacteria in VFDB and 100 human disease pathway genes in KEGG database, of which 16 genes were related to bacterial infectious diseases. In details, 53 genes were related to flagellar biosynthesis, flagellar basal body protein, flagellar motor switching, and chemotaxis; 32 type IV pili genes; 3 outer membrane usher protein genes; 24 iron/haeme uptake and utilisation relative genes; and 1 biofilm-controlling response regulator. 610A2^T harbours 2 haemolysin-encoding genes, *hlyB* and *hlyIII*, as well as the haemolysin activation/secretion protein gene *fhaC*, a transcriptional regulator of haemolysin *slyA*, and 3 HlyD family secretion proteins, which are consistent with the haemolytic activities on Columbia blood agar containing sheep blood.

The annotation results showed that 610A2^T may possess 4 types of secretion systems, consisting of 3 T3SS genes, 5 T4SS effectors, 1 T5SS gene, 19 T6SS genes (*tssA-H*, *tssK*, *tssL*, and *tssM*), concentrated in 2 regions of the chromosome. In addition, 610A2^T harbours 9 lipopolysaccharide biosynthesis genes; 11 haeme biosynthesis and utilisation genes; 4 superoxide dismutase genes (Fe-Mn and Cu-Zn families); 1 superoxide oxidase gene; 3 glutathione peroxidase genes; 2 metalloendopeptidase OMA1; 1 melanin-producing gene cluster containing MarR transcriptional regulator, 4-hydroxyphenylpyruvate dioxygenase, and homogentisate 1,2-dioxygenase; 3 catalase genes; 12 alginate biosynthesis and regulation genes; 2 nitrate reductase genes; 1 isocitrate lyase gene; and 1 succinate dehydrogenase gene.

3.4 AST and related genes

Owing to the slow growth rate of 610A2^T, its AST could not be tested using the BD Phoenix-100 equipment (BD, Sparks, MD, USA) with BD PhoenixTM NMIC 413 panels. Among the drugs detected with CHNENF and MYCOTB plates, 610A2^T was sensitive to 10 drugs: azithromycin (8 μ g/mL), chloramphenicol (16 μ g/mL), tetracycline (8 μ g/mL), streptomycin (16 μ g/mL), tigecycline (0.5 μ g/mL), ofloxacin (1 μ g/mL), moxifloxacin (0.06 μ g/mL), and rifampin (0.12 μ g/mL), with amikacin (<4 μ g/mL) and colistin (<0.25 μ g/mL) being the most sensitive. 610A2^T was resistant to ertapenem (8 μ g/mL) and rifabutin (0.5 μ g/mL). Fifteen drugs exceeded the maximum detection concentrations of the assay: cycloserine (>256 μ g/mL), kanamycin (>40 μ g/mL), para-aminosalicylic acid (>64 μ g/mL), ethionamide (>40 μ g/mL), isoniazid (>4 μ g/mL), ethambutol (>32 μ g/mL), cefotaxime (>16 μ g/mL), ceftazidime (>32 μ g/mL), ceftazidime/avibactam (>16/4 μ g/mL), meropenem (>2 μ g/mL), ampicillin/sulbactam (>32/16 μ g/mL), ciprofloxacin (> 0.12 μ g/mL), nalidixic acid (> 32 μ g/mL), ampicillin (> 32 μ g/mL), and trimethoprim-sulfamethoxazole (TMP-SMX, > 8/152 μ g/mL).

Comparison against the ARDB, CARD, and KEGG databases revealed that 610A2^T carried 41 antibiotic-resistant relative genes on its genome, including 12 genes, *aac6-I*, *acrb*, *adeb*, *ceob*, *emre*, *macb*, *mexc*, *mexe*, *oprn*, *smed*, *smee*, and *ykkd*, coding for multi-drug resistance efflux pump for chloramphenicol, aminoglycoside, fluoroquinolone, macrolide, and tetracycline resistance; extended-spectrum beta-lactamase (ESBL) gene *blaCTX-M*, subclass B3 metallo- β -lactamase gene *blaB*, and *pbp1a* and *pbp2b* for cephalosporin, penicillin, and β -lactam resistance; *rosA* and *rosB* for fosmidomycin resistance; as well as some special resistant genes, *bacA* for bacitracin, *ksgA* for kasugamycin, *dfrA-26* for trimethoprim, and *vanA* for vancomycin and teicoplanin.

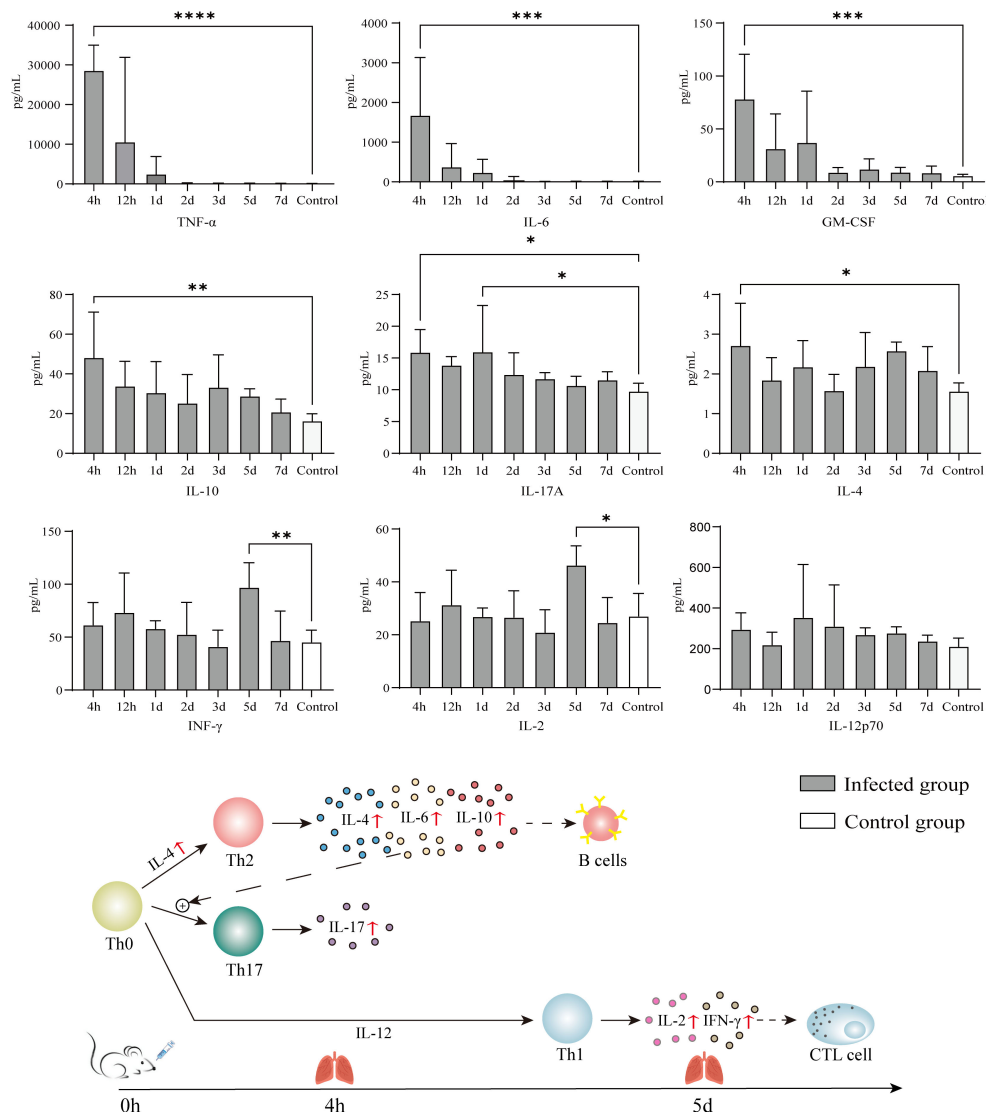


FIGURE 4

Immune response of KM mice intranasally infected with 610A2^T. At 4 h, 12 h, 1 day, 2 days, 3 days, 5 days, and 7 days post-infection, the mice were sacrificed and 9 cytokines, TNF-α, GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12p70, and IL-17A, in the lung homogenates were measured using Luminex. Values are mean ± SD (n = 10) obtained from 2 independent experiments. One-way ANOVA followed by Bonferroni's multiple comparison post-test were used for statistical testing, and the groups with significant differences when compared with the control were marked with asterisks.

* $P < 0.03$, ** $P < 0.002$, *** $P < 0.0002$, **** $P < 0.0001$.

3.5 Systematic evaluation of physiological and chemotaxonomic properties

610A2^T grew in all 4 tested media. The colonies on nutrient agar were translucent, smooth, and moist, with brown water-soluble pigment (Figure 5A); on blood agar, they were greyish white with a stimulating ammonia odour and a haemolytic ring (Figure 5B). The strain grew under conditions of 15–37 °C, pH 6–8, and NaCl concentration of 0–2%, but it did not grow below 4 °C and above 40 °C and did not tolerate 5% salt concentration like *S. maltophilia* and *S. terrae*. During semi-solid puncture culture, bacteria grew diffusely along the puncture line; however, no pigment was produced, indicating motility; pigment production was aerobic. 610A2^T was rod-shaped gram-negative, and without spores or

capsules. Under electron microscopy, the bacterial body was about $1.5\text{--}2 \times 0.5 \mu\text{m}$ with a single extreme flagella (Figure 5C).

610A2^T grew slower than *S. maltophilia* strains in TSB. After 48 h, the cultures of 610A2^T showed turbidity, but the entire growth curve was flat without a logarithmic phase (Figure 6A). Although the growth rate was slow, the biofilm index showed that 610A2^T had a strong biofilm-forming ability, which was significantly higher than that of all *S. maltophilia* strains after 3 h of cultivation (Figure 6B).

The biochemical results showed that, in the API 20NE test, 610A2^T was positive for nitrate reduction, hydrolysis of quercetin and gelatin, assimilation of *N*-acetylglucosamine, maltose, and citric acid. In API ZYM, it was positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, and

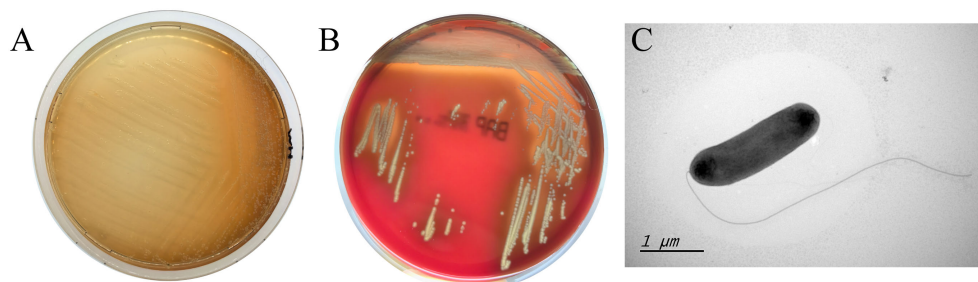


FIGURE 5

Clones of 610A2^T on MH medium (A), columbia blood agar (B) and its transmission electron microscopy image (C). Obvious brown water-soluble pigment and haemolytic ring can be observed on MH medium and blood agar, respectively. The bacterial body of 610A2^T is about 1.5–2 × 0.5 μm with a single extreme flagella.

naphthol-AS-BI phosphate hydrolase. In API 50CH, only starch fermentation was detected. The major differentiating feature of 610A2^T was that it could not assimilate glucose and mannose, but it could ferment starch.

In the MALDI-TOF spectrum, the 4 main peaks of 610A2^T were located at 5254.065 Da, 4857.675 Da, 2770.713 Da, and 6118.187 Da, of which the 4857 Da peak was genus-specific and the 2770.713 Da peak was 610A2^T-specific. In the local Bruker_MSP library, only 5 *Stenotrophomonas* species were found: *S. acidaminiphila*, *S. maltophilia*, *S. pictorum*, *S. rhizophila*, and *S. nitritireducens*. The identification scores of 610A2^T for these species ranged from 1.625 to 1.370, with the highest similarity to *S. pictorum*. These data

indicate that 610A2^T is a distinct species closely associated with these species of *Stenotrophomonas*.

The predominant fatty acids of 610A2^T were iso-C_{15:0}, iso-C_{14:0}, summed Feature 3 (C_{16:1}ω7c/16:1ω6c), iso-C_{16:0}, and C_{15:0} anteiso and sum in Feature 9 (iso-C_{17:1}ω9c). It contained all 3 fatty acids, iso-C_{11:0}, iso-C_{11:0} 3OH, and iso-C_{13:0} 3OH, characteristic of the genus *Stenotrophomonas*, but no matches were found in RTSBA6. 610A2^T also contained all characteristic fatty acids of *S. terrae* and *S. humi*: iso-C_{14:0} (14.2% and 15.7%), iso-C_{15:1} (4.6% and 2%), iso-C_{16:0} (8% and 12.7%), and iso-C_{17:1}ω9c (7.2% and 4.6%, respectively) (Heylen et al., 2007). The contents of these 4 fatty acids in 610A2^T were 15.45%, 1.5%, 11.9%, and 5.22%, respectively,

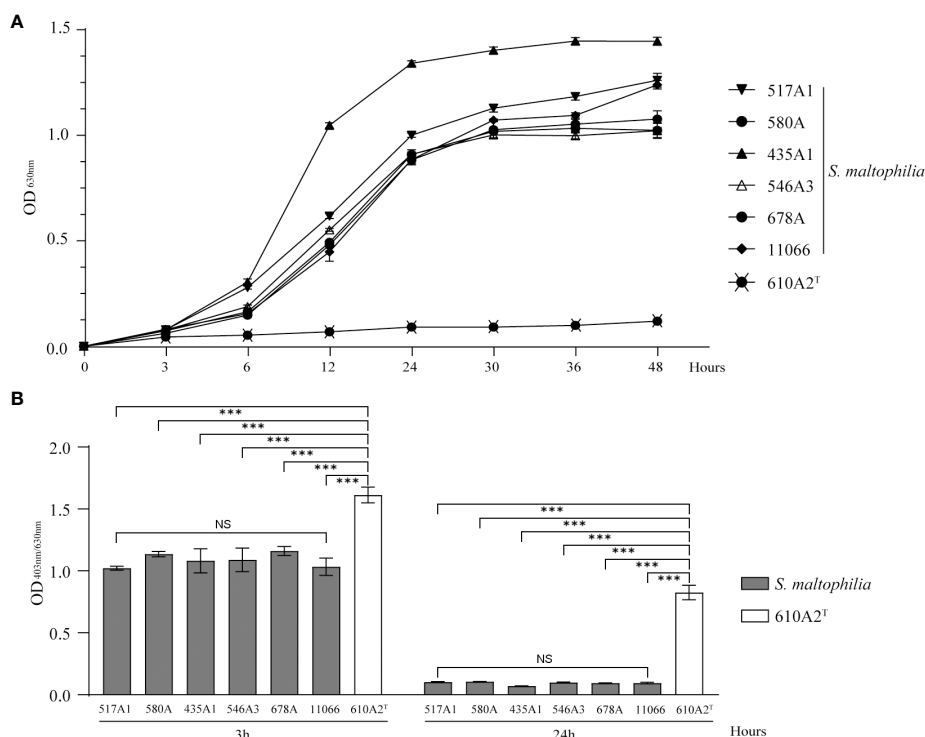


FIGURE 6

Growth curve (A) and biofilm formation (B) of 610A2^T and control strains in TSB medium. Significant differences between groups are marked with asterisks, ***P < 0.0002. NS, without statistical significance.

indicating that the similarity of 610A2^T to *S. humi* is higher than that to *S. terrae*.

4 Discussion

When evaluating the pathogenicity of strain 610A2^T, we used the outbred mouse KM, which has strong disease resistance and adaptability and can better reflect the genetic diversity of the human population. Within the first day post-infection, the bacterial load of 610A2^T in the lungs decreased sharply, and 610A2^T was cleared completely within 48 h. The lung clearance rate of 610A2^T in KM mice was faster than that of *S. maltophilia* in many inbred mouse strains, which also showed a larger decrease in CFU at 24 h, but could generally still be detected on day 3–7 post-infection and could even replicate in A/J mice with a transient increase in CFU at 4 and 8 h post-inoculation (Di Bonaventura et al., 2010; Rouf et al., 2011; Pompilio et al., 2018). After infection, both *S. maltophilia* (Di Bonaventura et al., 2010; Pompilio et al., 2018) and 610A2^T could be isolated in the spleen, suggesting that, at the same time of lung infection, *Stenotrophomonas* could disrupt the integrity of the lung epithelial barrier, cause bacteraemia, spread to other tissues, and pose an uncertain risk of assisting other pathogens in spreading and forming extrapulmonary lesions.

Combining with the acute infectious cytokine responses, the animal experiment in this study can confirm the pathogenicity of 610A2^T on the basis of the significant weight loss in the mice, tissue reactions due to inflammatory infection, and pulmonary congestion detected in the histopathological sections, although 610A2^T can be completely cleared from a healthy host. 610A2^T belonged to the *S. terrae* clade in the phylogenetic tree. In phylogenetic trees, whether constructed based on the concatenation of translated protein sequences (Patil et al., 2018) or the 16S rDNA gene, it is a clade that is relatively far from SMC. All species in this clade have been isolated from the environment and never been isolated from humans (Heylen et al., 2007). The isolation of 610A2^T changed the distribution of the pathogenic species. Together with *S. acidaminiphila* and *S. rhizophila*, all 3 main clades of the genus *Stenotrophomonas* have isolates from human sources.

610A2^T inherently possesses many pathogenic mechanisms, which can be inferred from the composition of functional genes on the genome, including those for motility (flagella and type IV pili), surface adherence (flagella, fimbriae, and LPS), damage capacity to host (protein secretion systems and extracellular enzymes), iron uptake and utilization, biofilm formation, protection against host defence (superoxide dismutase, hydroperoxidase, catalase, and melanin) as well as multi-antibiotic resistance (Di Bonaventura et al., 2010; Brooke, 2021). In addition, the genomic analysis suggested that 610A2^T produces type III haemolysin, for which erythrocyte lysis capacity and virulence have been confirmed in a mouse model infected with *Vibrio vulnificus* (Baida and Kuzmin, 1996; Chen et al., 2004; Moonah et al., 2014). In the genus *Stenotrophomonas*, colony pigmentation and brown diffusible pigments have been reported in only *S. maltophilia* (Romanenko et al., 2008; Liaw et al., 2010), *S. nitritireducens* (Finkmann et al., 2000), and *S. rhizophila* (Romanenko et al., 2008) isolates. Pigments and biofilm formation interact, and both are

components of bacterial pathogenicity, enabling the colonisation or infection of hosts and promoting the attachment of other pathogens (Tamma et al., 2022). 610A2^T showed stronger abilities than *S. maltophilia* in both aspects, indicating that its pathogenicity may not be weaker than that of *S. maltophilia*.

However, the strain-specific cytokine responses induced by 610A2^T may be difference from those induced by *S. maltophilia*. In this study, 9 cytokines were selected on the basis of their inflammatory responses in *S. maltophilia*-infected murine model and patients with TB (Di Bonaventura et al., 2010; Mvubu et al., 2018). Among these cytokines, early inflammatory markers TNF-α and IL-6 showed consistently high expression after 610A2^T and *S. maltophilia* infection, with IFN-γ, IL-4, and IL-10 exhibiting different or even opposite changes (McDaniel et al., 2020) (Table 2). In mice infected with *S. maltophilia*, characteristic changes in cytokines were the persistent hyperexpression of IFN-γ for more than 3 days and sustained significantly lower level of IL-4 when compared with the control group (Di Bonaventura et al., 2010). IFN-γ was released in the early stage, along with large amounts of TNF-α and IL-2 and was closely associated with the ratio of T cells. Blocking the PD-1/PD-L1 pathway inhibited the apoptosis-inducing effect of *S. maltophilia* on T cells. The pleotropic cytokine IL-4 is a product of Th2 lymphocytes and inhibits Th1 cell differentiation. Therefore, the immune response to acute infection by *S. maltophilia* is predominantly a Th1-type response (Rouf et al., 2011). *S. maltophilia* may participate in the activation of T cells and induce subsequent T-cell exhaustion by activating the PD-1/PD-L1 signalling pathway, as the concentration of cytokines is reduced in the later stages (Wang et al., 2021; Xu et al., 2023). In contrast, in the 610A2^T-infected KM mice, cytokines IL-4, IL-6, and IL-10 secreted

TABLE 2 The inflammatory responses of *S. maltophilia* and 610A2^T infected mice and TB patient.

Cytokines	Classification	<i>S. maltophilia</i>		610A2 ^T	TB patient ³
		A/J mice ¹	DBA/2 mice ²	KM mice	
TNF-α	Pro-	*↑	*↑	*↑	*↑
IFN-γ	Pro-	*↑	*↑	*↑	*↑
GM-CSF	Adaptive	–	–	*↑	*↑
IL-1β	Pro-	–	*↑	–	ns
IL-2	Adaptive	–	ns↓↑	*↑	*↑
IL-4	Adaptive	–	*↓	*↑	*↑
IL-5	Adaptive	–	ns↑↓	–	ns
IL-6	Pro-	*↑	*↑	*↑	*↑
IL-10	Anti-	–	ns↓↑	*↑	*↑
IL-12	Anti-	–	*↑	ns	*↑
IL-17A	Pro-	–	–	*↑	*↑

¹ Data from (Rouf et al., 2011); ² Data from (Di Bonaventura et al., 2010); ³ Data from (Mvubu et al., 2018). * cytokines having significantly different between infected and control mice; ns without statistical significance; – no included in study.

↑ the concentration of cytokine in the infection group is higher than that in the control group; ↓ during the observation period, the concentration of factors in the infection group is initially lower than that in the control group, and then increase to a level higher than that in the control group.

by Th2 cells showed a rapid and significant increase; there was no difference in IL-12, the main cytokine that induces Th1 cell differentiation and inhibits Th2 cells, indicating that the immune response of 610A2^T was Th2 type (Figure 4). In addition, the lagging release of IFN- γ and IL-2 in 610A2^T-infection indicated that Th1 type was disinhibition in the later stage and also involved in the host's defence against the pathogen. In-depth research of the pathogenic *Stenotrophomonas* species including 610A2^T in same mouse strain infection model will further elucidate pathogenic and immunological characteristics.

Moreover, 610A2^T is resistant to half of the commonly used anti-TB drugs, including isoniazid, and carries ESBL and metallo- β -lactamase, which can hydrolyse all bicyclic β -lactam antibiotics (Hinchliffe et al., 2023). The co-existence of *Stenotrophomonas* may affect therapeutic efficacy in patients with TB. The AST results showed that all drugs recommended by the Infectious Diseases Society of America for the treatment of *S. maltophilia* infection (Tamma et al., 2022) may be applicable to 610A2^T, except TMP-SMX, which is recommended as the first-line agent for the treatment of *S. maltophilia* infection owing to the low isolation rate of resistant strains (Chang et al., 2015; Gröschel et al., 2020). A combination of colistin and rifampicin may be used for patients with mixed 610A2^T and TB infections.

4.1 Description of *Stenotrophomonas pigmentata* sp. nov.

Stenotrophomonas pigmentata sp. nov. (pig.men.ta'ta. L. fem. adj. *pigmentata*, pigmented, coloured).

The type strain 610A2^T was isolated from patients with pulmonary TB. It is a Gram-negative bacillus with a single extreme flagella and can produce brown water-soluble pigment. Growth is observed at 15–37 °C, pH 6–8, and 0–2% NaCl concentration, but it does not grow below 4 °C, above 40 °C, and at 5% salt concentration. 610A2^T yielded positive results for nitrate reduction, quercetin and gelatin hydrolysis, and starch fermentation. The 4 main peaks in the MALDI-TOF spectrum were located at 5254.065 Da, 4857.675 Da, 2770.713 Da and 6118.187 Da. The predominant fatty acids are iso-C_{15:0}, iso-C_{14:0}, summed Feature 3 (C_{16:1}ω7c/16:1ω6c), iso-C_{16:0}, and C_{15:0} anteiso and sum in Feature 9 (iso-C_{17:1}ω9c). 610A2^T has multi-drug resistance and intrinsic resistance to β -lactams, carbapenems, and trimethoprim-sulfamethoxazole, harbouring several multi-drug resistance efflux pump and antibiotic resistant genes. This strain is pathogenic to mice.

The complete genome of type strain 610A2^T comprises 4681496 bp and GC content of 63.29%, and the complete genome and 16S rDNA gene sequence are deposited in GenBank under accession numbers CP130832.1 and OR936313, respectively. The type strain is 610A2^T (=GDMCC 1.4134^T =JCM 36488^T).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession

number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, CP130832.1, <https://www.ncbi.nlm.nih.gov/genbank/>, OR936313.

Ethics statement

The animal study was approved by the Laboratory Animal Welfare & Ethics Committee of the National Institute for Communicable Disease Control and Prevention (Issue number 2023-021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Investigation, Writing – review & editing. ZY: Investigation, Writing – review & editing. XF: Investigation, Writing – review & editing. DX: Investigation, Writing – review & editing. HL: Writing – review & editing. XZ: Investigation, Writing – review & editing. RW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Temperatures above 37°C increase virulence of a convergent *Klebsiella* *pneumoniae* sequence type 307 strain

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Background: Convergence of *Klebsiella pneumoniae* (KP) pathotypes has been increasingly reported in recent years. These pathogens combine features of both multidrug-resistant and hypervirulent KP. However, clinically used indicators for hypervirulent KP identification, such as hypermucoviscosity, appear to be differentially expressed in convergent KP, potential outbreak clones are difficult to identify. We aimed to fill such knowledge gaps by investigating the temperature dependence of hypermucoviscosity and virulence in a convergent KP strain isolated during a clonal outbreak and belonging to the high-risk sequence type (ST)307.

Methods: Hypermucoviscosity, biofilm formation, and mortality rates in *Galleria mellonella* larvae were examined at different temperatures (room temperature, 28°C, 37°C, 40°C and 42°C) and with various phenotypic experiments including electron microscopy. The underlying mechanisms of the phenotypic changes were explored via qPCR analysis to evaluate plasmid copy numbers, and transcriptomics.

Results: Our results show a temperature-dependent switch above 37°C towards a hypermucoviscous phenotype, consistent with increased biofilm formation and *in vivo* mortality, possibly reflecting a bacterial response to fever-like conditions. Furthermore, we observed an increase in plasmid copy number for a hybrid plasmid harboring carbapenemase and *rmpA* genes. However, transcriptomic analysis revealed no changes in *rmpA* expression at higher temperatures, suggesting alternative regulatory pathways.

Conclusion: This study not only elucidates the impact of elevated temperatures on hypermucoviscosity and virulence in convergent KP but also sheds light on previously unrecognized aspects of its adaptive behavior, underscoring its resilience to changing environments.

KEYWORDS

K. pneumoniae, temperature-dependent virulence, hypermucoviscosity, hypervirulence, plasmid copy number, transcriptomics

1 Introduction

The opportunistic pathogen *Klebsiella pneumoniae* is frequently associated with nosocomial infections worldwide including pneumonia, urinary tract, and bloodstream infections (Mathers et al., 2015). Classic, mostly nosocomial *K. pneumoniae* (cKp) often affects individuals with compromised immune systems (Lan et al., 2021) exacerbated by the rise of multidrug-resistant (MDR) representatives (Kochan et al., 2022).

Beyond the hospital walls, hypervirulent, *K. pneumoniae* (hvKp) strains can cause infections in healthy individuals (Choby et al., 2020). HvKp harbors a repertoire of key virulence factors such as siderophores (Russo and Marr, 2019). In recent years, the global emergence of converging pathotypes of *K. pneumoniae* strains contributed to difficult-to-treat infections, as they combine extensive drug resistance with hypervirulence mostly driven by hybrid plasmids (Shankar et al., 2022).

Traditionally, *K. pneumoniae* hypervirulence has been identified through a positive string test (Catalán-Nájera et al., 2017). This test explores “hypermucoviscosity”, a characteristic associated with better evasion of macrophages contributing to the invasive potential of hvKp (Russo et al., 2018). Despite the assumption that hypervirulence and hypermucoviscosity are connected, there is evidence that hypermucoviscosity is not a peculiar marker of hypervirulence (Lee et al., 2006). Precise determination of hypervirulence involves *in vivo* experiments and specific genetic markers like *iucA* (hydroxamate siderophore), *iroB* (catecholate siderophore), and *rmpA* or *rmpA2* (regulator of the mucoid phenotype) (Russo et al., 2018). Hypermucoviscosity correlates with clinical outcomes such as pyogenic liver abscesses (Shon et al., 2013). While more than 79 serologically defined capsular types exist, and genomics continuously reveal further diversity in these surface antigens, they differ in composition, which impacts virulence and hypermucoviscosity (Follador et al., 2016; Lam et al., 2022). Especially monosaccharides such as mannose and rhamnose seem to play a role (Huang et al., 2022). Other complex biosynthetic and regulatory mechanisms responding to external stimuli such as iron availability and carbon sources are also involved (Mike et al., 2021). While mechanisms for bacterial adaptation to host temperatures are well-established, the impact of temperature changes within the

host, such as fever episodes, on the regulation of virulence factors is not fully explored. Notably, there is a significant gap in understanding the temperature effects on hypermucoviscosity of *K. pneumoniae*, particularly above 37°C (Lam et al., 2014; Mandin and Johansson, 2020).

Here, we investigated capsule production and hypermucoviscosity in a convergent *K. pneumoniae* ST307 strain at different temperatures. By combining omics with *in vitro* and *in vivo* phenotypic experiments, we revealed temperature dependence of hypermucoviscosity and additional bacterial virulence, which are seemingly based on plasmid copy number (PCN)- and transcriptional changes.

2 Materials and methods

2.1 Bacterial strains (Table 1)

TABLE 1 Description of the investigated strain and its genetic properties, including Name, Species, Sequence type (capsule type), Plasmids and their main features.

Name	Species	ST (capsule type)	Plasmids and features
PBIO1953	<i>K. pneumoniae</i> (Heiden et al., 2020)	307 (KL102, O1/O2v2)	Plasmid 1 <i>bla</i> _{NDM-1} , <i>rmpA</i> Plasmid 2 <i>bla</i> _{CTX-M-15} Plasmid 3 Plasmid 4 <i>bla</i> _{OXA-48} Plasmid 5

2.2 Hypermucoviscosity

In the string test, a sterile loop was applied to a single colony on an agar plate, which had been cultured at varying temperatures (28°C, 37°C, 40°C, 42°C) for a duration of 24 h. The loop was gently lifted, and if there was a 5 mm extension without breaking, the string test was considered positive, which characterizes hypermucoviscosity (Fang et al., 2004). Furthermore, the hypermucoviscosity was confirmed with the sedimentation assay as described previously (Mike et al., 2021). In short, 50 µL of a standardized bacterial suspension (0.5 McFarland standard turbidity in 0.9% (w/v) aqueous NaCl solution) was transferred

into 5 mL LB broth and was incubated at the desired temperature (RT, 28°C, 37°C, 40°C, 42°C) for 24 h. Afterwards, 1.5 mL of the 24 h culture was transferred into a 2 mL reaction tube and centrifuged for 1000 x g for 5 minutes, 200 µL from the supernatant as well as from the 24 h culture were pipetted as triplicates into a 96 well plate and the OD₆₀₀ was measured with a plate reader (CLARIOstarplus, BMG Labtech, Ortenberg, Germany) (Eger et al., 2021).

The ability to generate exopolysaccharides was assessed using BHI agar plates supplemented with 5% sucrose (w/v) and 0.08% congo-red (w/v), following the methodology outlined in a prior study (Freeman et al., 1989). A single colony was picked up from an LB agar plate, streaked onto the stained BHI agar plates, and subsequently incubated overnight for 24 h at (RT, 28°C, 37°C, 38°C, 39°C, 40°C, 42°C). If the single colonies color black, they would be classified as positive for exopolysaccharides, while single colonies which are white to yellow would be considered negative for exopolysaccharide production.

2.3 Attachment and specific biofilm formation

The temperature influence on attachment factors was investigated with the crystal violet (CV) assay, as described previously (Schaufler et al., 2016; Eger et al., 2022). In short 50 µL of an overnight culture was transferred to 5 mL LB. After a visible turbidity samples were adjusted to an OD₆₀₀ of 0.01 and 200 µL was transferred as into a 96 well plate. Afterwards, they were incubated at 28°C, 37°C, 40°C and 42°C for 24 h. Following the incubation, the OD₆₀₀ was measured to determine the overall growth. Then, the supernatant of each well was discarded, the plate was washed three times with deionized water and dried for 10 min. Subsequently, fixed with 250 µL methanol for 15 min and then air dried for 15 min. Now the remaining cells were stained with 250 µL 1% crystal violet solution (w/v) for 30 min. This was followed by three washing steps with deionized water and drying for 10 min. The stained bacteria were dissolved with 300 µL of an ethanol acetone mixture with a ratio of 80 to 20 (v/v) and shaken for 30 min at 220 rpm at room temperature. Then the OD₅₇₀ was measured. The specific biofilm formation (SBF) (Niu and Gilbert, 2004) was then calculated. The negative control was subtracted from the OD₅₇₀ of the sample and then divided by the OD₆₀₀ of the sample.

2.4 *Galleria mellonella* in vivo infection model

To test the influence of different temperatures on the pathogenic potential the *Galleria mellonella* in vivo infection model was used, as previously described (Insua et al., 2013) with slight adjustments of the experiment setup in respect to this temperature dependent study. Briefly, 2 mL of overnight culture of PBIO1953 was harvested and pelleted at 16000 x g for 5 min at RT. The pellet was once washed with PBS and diluted to an OD₆₀₀ of 1.0. The 2 × 10⁹ CFU/mL were further adjusted to 2 × 10⁶ CFU/

mL. The larvae (from Peter Zoopalast, Kiel, Germany) were randomly divided into 6 different groups of 10 individuals. 10 µL of the bacterial suspension was injected into the right proleg of the larvae. For the control groups 10 µL of PBS solution was used as injection, to check if the infection of *Galleria mellonella* larvae was affected by the traumata or the altered incubation temperature. The sample and control groups were incubated in a 90 mm glass petri dish at 28°C, 37°C and 40°C each. The vital state of the larvae was controlled every 24 h. The larvae were considered dead, if they showed strong pigmentation accompanied by not responding to physical stimuli. This assay was performed three independent times the results were pooled for each condition. a Kaplan-Meier plot was generated where the survival was plotted to visualize the mortality rates of the *Galleria mellonella* at different temperatures.

2.5 Plasmid copy number

To determine the plasmid copy numbers of the three largest plasmids (plasmid 1 *bla*_{NDM-1} and *rpmA*, plasmid 2 *bla*_{CTX-M-15}, plasmid 3) at different temperatures (RT, 28°C, 37°C, 40°C, 42°C) qPCR was used. Therefore, sets of different Primers seen in [Supplementary Table 1](#) were designed to amplify a specific region on the gene constructs of interest. The primers were designed according to the manufacture's instruction for Luna qPCR Kit (New England Biolabs GmbH, Ipswich, MA, US) and ordered from Eurofins (Eurofins Genomics Europe Shared Services GmbH, Ebersberg, Germany).

The calibration for the plasmids (1–3) and the chromosome was performed with the Q5 High-Fidelity DNA Polymerase (New England Biolabs GmbH, Ipswich, MA, US) according to the manufacture's guideline, and isolated with NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and quantified with the Qubit 4 fluorometer using the dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR was performed with the Luna qPCR protocol (New England Biolabs GmbH, Ipswich, MA, US) in regard to the qPCR cyclor CFX Opus 96 Real-Time PCR System (Bio-Rad, Hercules, CA, US). Each sample was set up as seen in the [Supplementary Table 2](#) and each sample was measured in triplicates following the protocol shown in [Supplementary Table 3](#).

The measured concentration of the samples was related to the overall length of the gene structure and the plasmid copy number was calculated as

$$PCN = (P/C) * (Lc/Lp)$$

PCN: Plasmid copy number per genome equivalent P: Overall amount of the plasmid (in ng) C: Overall amount of the chromosome (in ng) Lc: Overall length of the chromosome (in base pairs) Lp: Overall length of the plasmid (in base pairs).

2.6 Electron microscopy

A single colony was inoculated into 30 mL of LB in an Erlenmeyer flask and cells were incubated at 28°C, 37°C, 40°C

and 42°C for 24 hours. Subsequently, the bacterial suspension was adjusted to $OD_{600} = 1$, and 1 mL of the suspension was diluted with 9 mL of 0.9% NaCl. The resulting suspension was then filtered through a hydrophilic polycarbonate filter (0.2 µm pore size, Merck Millipore). A segment of the filter was fixed with 1% glutaraldehyde and 4% paraformaldehyde in washing buffer (10 mM cacodylate buffer, 1 mM $CaCl_2$, 0.075% ruthenium red; pH 7) and then the samples were stored at 4°C until further processing (not more than 16 hours).

Samples were washed with washing buffer three times for 10 min each time, treated with 2% tannic acid in washing buffer for 1 h at RT, and then washed again with washing buffer three times for 15 minutes each time. Afterwards, the samples were dehydrated in a graded series of aqueous ethanol solutions (10, 30, 50, 70, 90, 100%) on ice for 15 min each step. Before the final change to 100% ethanol, samples were allowed to reach RT and then critical point-dried with liquid CO_2 . Finally, the samples were mounted on aluminum stubs, sputtered with gold/palladium, and examined with a field emission scanning electron microscope Supra 40VP (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany) using the Everhart-Thornley SE detector and the in-lens detector in a 70:30 ratio at an acceleration voltage of 5 kV. All micrographs were edited by using Adobe Photoshop CS6.

2.7 DNA isolation

The DNA isolation for the qPCR was performed by either 1 mL of 24 h cultures in LB at 28°C, 37°C, 40°C and 42°C or directly from the BHI agar plates supplemented with congo-red and sucrose incubated at the 4 different temperatures, where 4 to 6 single colonies were scraped off and resuspended via vortexing in 500 µL of PBS in a 1.5 mL test tube. The DNA isolation was then performed with the *MasterPure DNA Purification Kit for Blood* according to the manufacturer's specifications (Lucigen, Middleton, WI, USA). The quantification of the DNA was ensured by using the Qubit 4 fluorometer using the dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.8 RNA isolation and analyses

For transcriptomic analysis, the RNA was isolated from 50 mL liquid 24 h LB medium cultures ($n=3$) at 28°C, 37°C, 40°C and 42°C. 800 µL of the cultures were harvested in a 1.5 mL reaction tube and instantly chilled with liquid nitrogen for 5 s, to inhibit changes in the transcriptome. After the centrifugation ($16,000 \times g$ for 3 min at 2°C), the following RNA isolation was performed immediately with MonarchTM Total RNA Miniprep Kit (New England Biolabs GmbH, Ipswich, MA, US) according to the manufacture's instruction. The quality of the isolated RNA was tested using the Bioanalyzer with the Total RNA Nano Chip (Agilent, Santa Clara, CA, US). The samples were shipped on dry ice to Novogene (NOVOGENE COMPANY LTD., Cambridge, UK) for RNA sequencing (Illumina NovaSeq 6000; paired-end 150 bp).

The closed genome sequence of PBIO1953 was annotated with Bakta v. 1.7.0 and bakta database v. 5. In order to process the raw

reads and to further quality trim the data, Trim Galore v. 0.6.8 (<https://github.com/FelixKrueger/TrimGalore>) was used. The trimmed reads were then mapped with Bowtie 2 v. 2.5.1 (mode: –very-sensitive-local), where the assembly of PBIO1953 was used as a reference. The gene counts were calculated using featureCounts v.2.0.1/stranded-mode based on PBIO1953s' annotation. In the following step, the count table was imported into R v. 4.3.1 (<https://www.R-project.org/>), and differentially expressed genes were identified with DESeq2 v. 1.40.0 in default mode, with one exception; genes with rowSums of <10 in the count table were excluded before the analysis. Within our analyses, we used an absolute log2 fold change 1.5 thresholds combined with an adjusted e value lower than 0.05 to determine differences within gene expression, between the different incubation conditions. We excluded one replicate which was incubated at 37°C due to a shift on principal component 1 (PC1) and PC2, which can be seen in [Supplementary Figure S2](#) where principal-component analysis is plotted. Finally, all genes were annotated using the online tool egg-nog-mapper v. 2.1.9 to extract COG (cluster of orthologous groups) categories.

2.9 Statistical analysis

Statistical analyses were conducted using GraphPad Prism v. 5.5.1 for Windows, developed by GraphPad Software (San Diego, CA US). To assess qPCR results, the Bio-Rad CFX Maestro 2.3 v. 5.3.0 22.1030 (Bio-Rad, Hercules, CA, US) was utilized. All phenotypic experiments consisted of three or more independent biological replicates. Unless otherwise specified, data were presented as the mean and standard deviation. Statistical significance was determined using p values below 0.05 to indicate significant differences among the results.

2.10 Ethic statement

Ethical approval for this study was obtained from the ethics committee of the University of Greifswald, Germany (Approval number: BB 133/20), ensuring compliance with established ethical standards. Informed consent from patients was waived as samples were collected under a hospital surveillance framework for routine sampling. The study strictly adheres to the principles outlined in the Helsinki Declaration. No identifiable patient data, such as names and ages, was included in the study, and no tissue samples were utilized during isolation procedures.

3 Results

We investigated hypermucoviscosity and mortality for a previously published, convergent *K. pneumoniae* ST307 strain [PBIO1953 as described in [Table 1](#) ([Heiden et al., 2020](#))] at RT, 28°C, 37°C, 40°C, and 42°C ([Figure 1](#)). First, we stained capsular exopolysaccharides ([Figure 1A](#)). Here, white to yellow-beige colonies indicate “normal” exopolysaccharide production, which

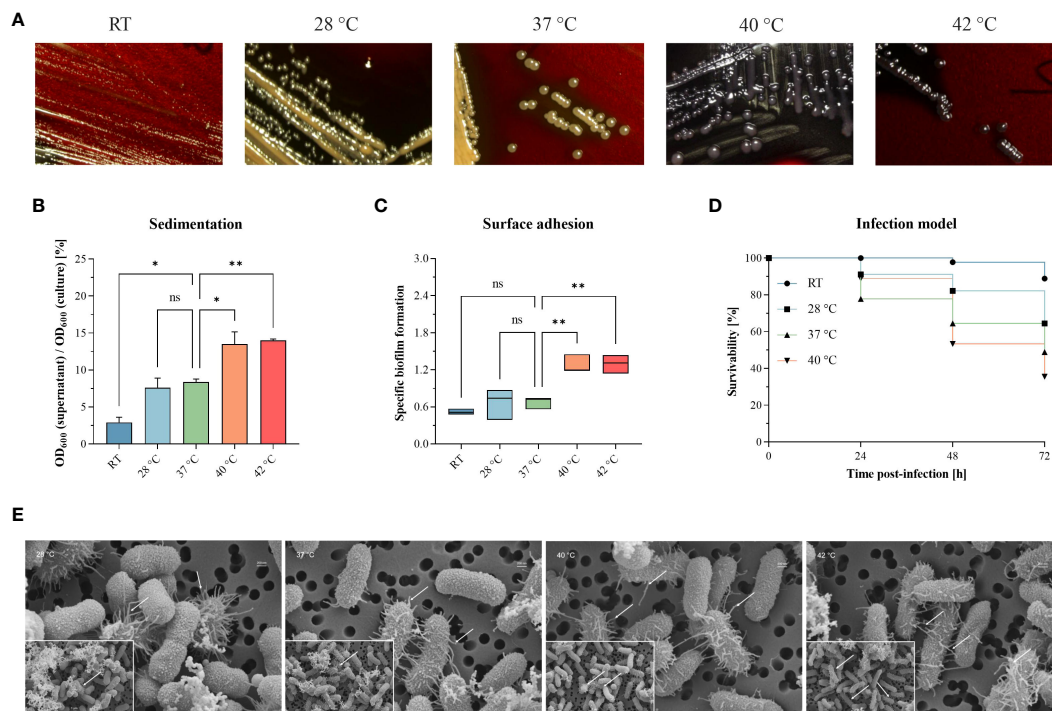


FIGURE 1

Different temperatures affect mucoviscosity and overall virulence of the convergent *K. pneumoniae* ST307 strain PBIO1953. (A) Staining of capsular polysaccharides revealed a temperature-dependent change from a “normal” mucoid phenotype (yellow-beige colonies) to a hypermucoid phenotype (black colonies) at 40°C and 42°C. (B) The hypermucoviscosity was associated with a decrease in sedimentation upon centrifugation at 1,000 x g for 5 min. Results are shown as mean and standard error of percentage OD₆₀₀ remaining in the supernatant after centrifugation (n = 3). (C) Temperatures above 37°C resulted in increased adhesion to plastic surfaces. Results are expressed as growth-adjusted specific biofilm formation. The line in the box indicates the median value (n = 3). (D) The *in vivo* infection model showed a temperature-dependent increase in mortality rates. Kaplan-Meier plot of survival rates of the *G. mellonella* larvae (n = 30). Results are expressed as mean percent of survivability after injection of 10⁵ CFU/larvae. For all results, mucoviscosity-associated characteristics at the different temperatures were compared to 37°C using analysis of variance (one-way ANOVA with Dunnett’s multiple comparison *post hoc* test); ns, not significant; P* < 0.05; **P < 0.01. RT, room temperature. (E) Scanning electron micrographs of PBIO1953 at 28°C, 37°C, 40°C, and 42°C at 20,000x magnification, scale bar = 200 nm (inserts: 10,000x magnification, scale bar = 1 µm), arrowheads show fimbriae-like structures.

applied to PBIO1953 at RT, 28°C, and 37°C. In contrast, black colonies appeared above 37°C (Figure 1A; Supplementary Figure S6), implying increased polysaccharide biosynthesis (Freeman et al., 1989). String tests confirmed these result (data not shown), hypermucoviscosity was identified at 40°C and 42°C. The sedimentation assay also confirmed higher viscosity at 40°C ($p = 0.0325$) and 42°C ($p = 0.0187$) compared to 37°C, indicating hypermucoviscosity (Figure 1B). During our examination of a control for hvKp and a non-mucoid strain, we found no evidence of temperature dependence in contrast to PBIO1953, as illustrated in Supplementary Figure S7. Third, biofilm experiments revealed that increasing temperatures led to higher affinity of PBIO1953 to adhere to abiotic surfaces (Figure 1C). A significant increase in specific biofilm formation was observed at 40°C ($p = 0.0053$) and 42°C ($p = 0.0049$) in comparison to 37°C. Interestingly, this was not related to curli or cellobiose production (Supplementary Figure S1).

Finally, to explore the impact of different temperatures on the overall virulence of PBIO1953, we assessed mortality rates in *Galleria* (*G.*) *mellonella* larvae (Figure 1D). After 24 hours, mortality rates at 37°C (22.22%) exceeded those observed at 40°C (11.12%). However, 48 hours post-infection, mortality rates at 40°C consistently exceeded those at 37°C, with a peak after 72 hours. As

control, the larvae were mock-infected with PBS and incubated at RT, 28°C, 37°C, 40°C with no detected temperature influence on the larvae mortality (Supplementary Figure S3). At all temperatures tested, the death of the larvae was always accompanied by a dark brown to blackish coloration of the carcass, which indicates the activity of the larvae’s immune system before death (Pereira et al., 2020). Furthermore, there were no signs of an active immune system in the mock-infected larvae incubated at different temperatures (RT, 28°C, 37°C, 40°C). Note that incubation of mock-infected *G. mellonella* larvae at 42°C resulted in mortality rates greater than 10% [data not shown e.g. (Ménard et al., 2021)].

Interestingly, scanning electron micrographs indicate an increasing amount of virulence- and biofilm-associated fimbriae structures at elevated temperatures of 40°C and 42°C compared to lower temperatures of 28°C and 37°C (Figure 1E). The fimbriae-like structures appear as thin, filamentous appendages extending from the bacterial surface. These structures exhibit a thread-like morphology, characterized by their elongated and slender appearance. They are typically distributed across the bacterial surface, appearing as protrusions or projections that emanate from the cell wall. The presence of these structures is marked by the placement of small white arrows in the micrographs, indicating their location and

abundance. While individual fimbriae may vary in length and thickness, their collective presence forms a distinctive pattern across the bacterial surface. It is important to note that the bacterial capsule appears compromised during the staining process. Nevertheless, the visible increase of these structures at higher temperatures (40°C, 42°C) implies a temperature-associated regulation (Chen et al., 2011).

3.1 Different temperatures affect PCN

To explore the underlying mechanisms, PCN-variations were measured using qPCR. Previously, we have shown that the convergent PBIO1953 strain harbors five different plasmids, one of which is a hybrid plasmid (plasmid 1) encoding both AMR and hypervirulence genes (Heiden et al., 2020). The three largest PBIO1953 plasmids were included in the subsequent analysis: plasmid 1 (360,596 bp), positive for the metallo- β -lactamase gene *bla_{NDM-1}* and the regulator of the mucoid phenotype *rmpA*, plasmid 2 (130,131 bp) encoding the extended-spectrum β -lactamase (ESBL) gene *bla_{CTX-M-15}*, and plasmid 3 (72,679 bp) without any resistance genes (Figure 2).

As the hypermucoviscosity switch was observed above 37°C, we normalized all PCN to 37°C. Interestingly, the PCN differed not only based on the different temperatures but also regarding the respective plasmid and experimental set-ups (Figure 2). For the hybrid plasmid 1, the PCN increased with higher temperatures, with the highest value obtained at 42°C. The PCN of plasmid 2 did not show a clear temperature decency, but a PCN reduction was apparent at both 28°C and 42°C in the liquid culture set-up. Plasmid 3 showed a PCN decreasing with higher temperatures.

3.2 Temperature-dependent transcriptomic changes

For transcriptomics, we first focused on genes displaying differential gene expression (DGE) at 28°C, 40°C and 42°C (e-value < 0.05, $|\log_2\text{fold}|$ change > 1.5) in comparison to 37°C. DGE appeared mostly between 28°C and 37°C (Supplementary Figures S4, S5), while, at 37°C compared to 40°C and 42°C, we only noticed few differentially

expressed genes. Our initial hypothesis that *rmpA* would be differentially expressed was rejected and, genes related to the capsular gene cluster were unaffected.

When comparing 28°C-associated gene expression to 37°C (and 40°C and 42°C), we observed various, significant changes. Notably, the chromosomally located *ibpAB* gene and two small heat shock proteins encoded on plasmid 2, exhibited differential expression at increased temperatures. These genes are known for mitigating growth defects under thermal stress (Kitagawa et al., 2000). Furthermore, we observed the upregulation of several stress-related genes at 37°C and higher, including the Fur repressor, which possesses protective properties against reactive oxygen species (Achenbach and Yang, 1997) and *sodA*, a gene involved in radical scavenging. Of particular interest was the upregulation of *acrAB*, known for its role in antimicrobial resistance and virulence (Padilla et al., 2010). Remarkably, the expression of *mrkABCD*, which encodes the type 3 fimbriae system, increased at 37°C and above. Previous research has suggested that environmental factors (Johnson et al., 2011), like temperature, can impact attachment factors, making this observation particularly intriguing. Conversely, we noted a downregulation of *fimHGFDCA*, responsible for the expression of fimbriae type 1 (Struve et al., 2008), and the downregulation of the *rfb* gene, which encodes O-antigens (Kelly and Whitfield, 1996). Additionally, *ompA*, a known virulence factor and key element in immune system recognition (Llobet et al., 2009) was downregulated at increased temperatures. On plasmid 1, the partitioning system *parAB* responsible for segregation and plasmid stability (Bignell and Thomas, 2001) demonstrated increased expression at higher temperatures. Notably, genes associated with transposon activation and IS sequences were also upregulated on plasmid 1. The gene *iutA*, encoding aerobactin and contributing to bacterial virulence, was upregulated at 37°C (and 40°C and 42°C). Moreover, on plasmid 2, the metallo-resistance gene *arsR* was upregulated (Xu et al., 1996). Finally, plasmids 4 and 5 showed increased expression of genes involved in conjugative transfer, including *traA* (Yang et al., 2007) and *mobC* (Zhang and Meyer, 1997). When comparing the transcriptomes of 40°C to those at 37°C, DGE was observed in six genes. Notably, two downregulated genes, *metR* (associated with the methionine pathway) and *argC* (linked to the arginine pathway), were particularly interesting as they are important for general growth. Only two genes were

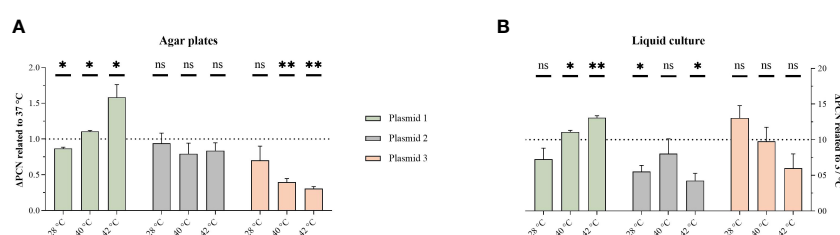


FIGURE 2

Temperature variation affect PCN. The PCN was determined from individual colonies grown on congo-red-dye-enriched agar plates (A) or from cells pelleted from sedimentation assay cultures (B). The results are expressed as mean and standard error (one sample t-test in comparison to 1 at 37°C ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

upregulated, with *betB* standing out as this gene is involved in the biosynthesis of osmoprotective choline-glycine betadine (Landfald and Ström, 1986). Similarly, the comparison of 42°C to 37°C revealed six differentially expressed genes. *BetB* demonstrated concordance with the upregulated genes at 40°C. An intriguing finding was the upregulation of *pecM*, a gene previously implicated as a potential deactivator of the *fim* operon and a member of the permease of the drug/metabolite transporter (DMT) superfamily.

4 Discussion

Hypermucoviscosity is often referred to as the most important virulence characteristic of hvKp. However, it is largely unclear how external stressors can affect capsule expression and thus virulence in bacteria, especially in convergent *K. pneumoniae*. We revealed that a convergent *K. pneumoniae* ST307 outbreak strain (Heiden et al., 2020), changed from a “normal” to a hypermuroid and pronounced virulent phenotype upon temperature exposure above the healthy human body temperature at about 37°C.

Bacterial capsule formation is a highly complex process influenced by several internal and external factors. Although hvKp frequently exhibits capsule types K1 and K2, a clear correlation between the capsule type and hypervirulence has not yet been established (Pan et al., 2008). To our knowledge, we are the first to report a temperature-dependent phenotypic switch for *K. pneumoniae*. Previously, Le et al. (2022) have shown that temperatures below 37°C may affect hypermucoviscosity, based on the presence of plasmid-encoded *rmpA* at 37°C when compared to RT (Le et al., 2022). In contrast, our results suggest that *rmpA*, although plasmid-encoded, was not differentially expressed at different temperatures. More importantly, the increased PCN plasmid 1 does seemingly not have any effect on *rmpA* expression, although it is known that higher gene abundance as a result of increased PCN can lead to higher gene expression. It seems likely that hypermucoviscosity does not exclusively depend on *rmpA* expression. This assumption has been previously supported by studies where *K. pneumoniae* isolates harbor impaired capsule locus genes and the respective regulator but show hypermucoviscosity (Dey et al., 2022).

While a substantial number of genes exhibited DGE between 28°C and 37°C, only a limited number of genes was differentially regulated at 37°C vs. 40°C or 42°C. For all comparisons, most of the differentially expressed genes were chromosomally encoded, which suggests that increasing temperatures up to pathophysiological conditions (i.e., fever) does not lead to major shifts in the transcriptome and to the phenotypic switch. Rather, this seems to fine-tune the expression of a small number of genes together with the different PCN. We surmise that temperature-dependent stress regulators and heat shock proteins may be involved in the regulation of capsule production, which is supported by previous studies (Dorman et al., 2018). However, why the upregulation of stress response regulators and proteins, which normally control the expression of a large number of protein-associated genes (Kitagawa et al., 2000), does not lead to a clearer change in the bacterial

transcriptome, remains unclear. The only direct regulatory pathway previously connected to capsule synthesis and influenced by increased temperatures was the *rcaA* gene, which contributes to capsular overexpression and is usually expressed at temperatures below 37°C (McCallum and Whitfield, 1991). Note that this is contrary to our findings.

Here, we identified a link between hypermucoviscosity and fimbrial regulation. The upregulation of type 3 fimbriae at temperatures above 28°C might be responsible for the fimbrial-like structures at higher temperatures as visualized by scanning electron microscopy. Interestingly, Lin et al. (2011) showed that hypermucoviscosity and type 3 fimbriae production can be co-regulated and depend on iron availability, and that iron limitation results in a reduction of mucoviscosity and fimbriae expression (Lin et al., 2011). Conversely, we observed a downregulation of type 1 fimbriae at higher temperatures. This might be because type 1 and type 3 fimbriae are independently regulated. However, in the CV assay, which measures specific biofilm formation, increased attachment at 40°C (and 42°C) compared to 37°C was measured, indicating a higher cell-to-cell and cell-to-plastic adherence, which could be fimbriae type 3-related and is essential in the transition from a planktonic to a multicellular lifestyle.

We speculate that PBIO1953 has evolved specific mechanisms to respond to the host's immune system, combined with a temperature-dependent formation of a physical capsule barrier and increased attachment at 40°C and 42°C. In the *in vivo* infection model, higher mortality was initially achieved after 24 hours at 37°C than at 40°C, which may be due to the initial encapsulation process combined with higher adherence at 40°C. As a result, the immune system response may be impeded, enabling bacterial proliferation. After 48 h and 72 h, we noticed a subsequent mortality increase at 40°C, again hinting towards better bacterial proliferation, possibly due to encapsulation and the previously mentioned changes. Similar has been previously suggested for various pathogens (Finlay and McFadden, 2006) but not *Klebsiella*. At the same time, we must acknowledge that our results are based on RNA sequencing data with stringent cut-offs and obtained from 24 h liquid cultures, which adds uncertainty when trying to apply these findings to *in vivo* situations. However, the general influence of the temperature on the *Klebsiella* strain could be observed and thus make general assumption on the *in vivo* situation possible. A further limitation is the fact that we only investigated a single *K. pneumoniae* strain. Prospective studies will have to explore whether similar applies to other strains. Nevertheless, our results suggest that enhanced temperatures and thus pathological conditions (i.e., fever) can lead to increased bacterial virulence, further exacerbating the overall tense situation. However, hypermucoviscosity could also represent a vulnerability that could be considered in a future treatment strategy.

We show that increased temperatures and thus potentially pathological conditions led to increased virulence in a convergent *K. pneumoniae* strain, which might play a pivotal role in shaping the dynamics of infection processes. In addition, our study contributes to a better understanding of the underlying mechanisms leading to hypermucoviscosity and *in vivo* bacterial virulence.

Data availability statement

The transcriptomic data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB72193 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB72193>). Additional genomic data of PBIO1953 are available under accession number PRJEB37933 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB37933>).

Ethics statement

The studies involving humans were approved by ethics committee of the University of Greifswald, Germany (Approval number: BB 133/20). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Ethical approval for this study was obtained from the ethics committee of the University of Greifswald, Germany (Approval number: BB 133/20), ensuring compliance with established ethical standards. Informed consent from patients was waived as samples were collected under a hospital surveillance framework for routine sampling. The study strictly adheres to the principles outlined in the Helsinki Declaration. No identifiable patient data, such as names and ages, was included in the study, and no tissue samples were utilized during isolation procedures. The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

JM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. MiS: Data curation, Formal analysis, Software, Writing – review & editing. LS: Formal analysis, Methodology, Writing – review & editing. SH: Data curation, Formal analysis, Software, Writing – review & editing. RS: Formal analysis, Investigation, Methodology, Writing – review & editing. MaS: Formal analysis, Writing – review & editing. JB: Formal analysis, Writing – review & editing. KB: Formal analysis, Writing – review & editing. EI: Formal analysis, Writing – review & editing. SG: Formal analysis, Writing – review & editing. EE: Writing – review & editing, Formal analysis, Investigation, Methodology, Validation, Writing – original

draft. KS: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1411286/full#supplementary-material>

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Molecular epidemiology of string test-positive *Klebsiella* *pneumoniae* isolates in Huzhou, China, 2020–2023

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Objective: This study used whole-genome sequencing (WGS) to explore the genetic diversity, virulence factors, and antimicrobial resistance determinants of string test-positive *Klebsiella pneumoniae* (KP) over a 4-year surveillance period in Huzhou, China.

Methods: In total, 632 clinical isolates were collected via hospital surveillance from 2020 to 2023; 100 were positive in the string test and these 100 strains were subjected to antimicrobial susceptibility testing using an agar dilution method followed by WGS.

Results: The resistance rates to cefotaxime (77.0%), trimethoprim-sulfamethoxazole (67.0%), and nalidixic acid (64.0%) were high. Multilocus sequence typing revealed high genetic diversity; there were 33 sequence types (STs) and 15 capsular serotypes. The most common ST was ST23 (16.0%) and the most common capsular serotype was K1 (22.5%). Virulome analysis revealed among-strain differences in virulence factors that affected bacterial adherence, efflux pump action, iron uptake, nutritional factors, metabolic regulation, the secretion system, and toxin production. The Kleborate strain-specific virulence scores of all 100 string test-positive KPs were derived: 28 strains scored 5, 28 scored 4, 21 scored 3, 12 scored 1, and 11 scored 0. All 77 strains with scores of 3 to 5 contained the *iucA* gene. The phylogeny based on whole-genome single nucleotide polymorphisms (wgSNPs) indicated high clonality; the string test-positive KP strains were grouped into six clades. Closely related isolates in each genetic cluster usually shared STs.

Conclusion: The present study highlights the significance of the KP *iucA* gene in terms of hypervirulence and the diverse genotypes of string test-positive KP strains isolated in Huzhou hospitals.

KEYWORDS

string test-positive KP, hypermucoviscosity, virulence, whole genome sequencing, molecular epidemiology

Introduction

Klebsiella pneumoniae (KP) is an increasingly significant pathogen associated with severe life-threatening diseases of various organs (Russo and Marr, 2019). It is an opportunistic Gram-negative pathogen that causes both nosocomial and community-acquired infections (Choby et al., 2020) including pneumonia, bacteremia, urinary tract infections, and others (Fazili et al., 2016). Hypervirulent KP (hvKP) is linked to an “invasive syndrome” that was first identified in Taiwanese patients with liver abscesses in 1986; hvKP is more virulent than “classical KP” (cKP) (Liu et al., 1986). Unlike cKP, hvKP exhibits hypermucoviscosity on blood plates. At the molecular level, the unique hvKP virulence genes promote both invasion and transmission, associated with multi-site and secondary metastatic infections. HvKP infection is linked to severe pathogenicity and high mortality (Shon et al., 2013; Fazili et al., 2016).

As sequencing costs decrease, bacterial whole-genome sequencing (WGS) has become more common. WGS rapidly generates accurate data that aid typing, phylogenetic analyses, and exploration of bacterial virulence and resistance characteristics (Ribot et al., 2019). WGS is increasingly used by public health laboratories that engage in pathogen surveillance; it is replacing conventional technologies.

The definition of hvKP is based on a combination of clinical and microbiological characteristics that differ from those of cKP (Harada and Doi, 2018). Not all hvKP strains are hypermucoviscous; certain cKP strains exhibit this feature (Russo and Marr, 2019). Hypermucoviscosity (associated with hypervirulence) reflects overexpression of capsular polysaccharides (Anantharajah et al., 2022). Experimentally, *iuc* and/or *rmpA* or *rmpA2* are the best hypervirulence markers; if they are absent, hypervirulence is lacking (Russo and Marr, 2019).

String test-positivity indicates hypervirulence. This study collected 632 KP strains from sentinel Huzhou hospitals from 2020 to 2023. In total, 100 hypermucoviscous strains were identified using the string test and subjected to antimicrobial susceptibility testing (AST) and WGS. Antimicrobial resistance (AMR) profiles, capsular serotypes, sequence types, antimicrobial resistance and virulence genes, and evolutionary relationships were evaluated. This comprehensive genomic analysis explored the genetic diversity, virulence potential, and AMR profiles. Our results aid the understanding of string test-positive KP prevalence, could help guide rational drug prescription, and may enhance the control of nosocomial infections in Huzhou.

Materials and methods

Strain collection

In all, 632 KP isolates were collected from six sentinel hospitals of the Chinese Pathogen Identification Net of Huzhou from 2020 to 2023; the samples included sputum, urine, secretions, and blood. After suspicious colonies were isolated, species were identified using

matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (bioMérieux, France). The strains were stored in glycerol broth at -70°C . *Escherichia coli* ATCC29522 was from the Chinese Center for Disease Control and Prevention.

String test

The hypermucoviscous phenotype was identified through a positive string test. All 632 isolates were tested. After a single colony grown overnight on a Columbia blood agar plate at 36°C was stretched using an inoculation loop, the formation of a viscous string > 5 mm in length identified a hypermucoviscous strain (Kumabe and Kenzaka, 2014).

Antimicrobial susceptibility testing

Eight classes of 17 antimicrobial agents (Thermo, USA) were used for AST. The minimum inhibitory concentrations (MICs) of chloramphenicol (CHL), trimethoprim-sulfamethoxazole (SXT), colistin (CT), ertapenem (ETP), meropenem (MEM), cefotaxime (CTX), ceftazidime (CAZ), ceftazidime/avibactam (CZA), tetracycline (TET), tigecycline (TIG), ciprofloxacin (CIP), nalidixic acid (NAL), azithromycin (AZM), amikacin (AMI), streptomycin (STR), ampicillin (AMP), and ampicillin/sulbactam (AMS) were detected using the agar dilution and broth microdilution methods. Breakpoint interpretations followed the 2020 guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020). Negative and blank controls were included; *Escherichia coli* ATCC 25922 served as a control strain.

Whole genome sequencing

All string-positive strains were subjected to WGS. DNA was extracted from overnight cultures using QIAamp DNA Mini Kits (Qiagen, Germany) following the manufacturer's instructions and DNA concentrations were determined using the Qubit 4 method (Thermo, USA). Quality-confirmed DNA was stored at -80°C until further use. WGS libraries were constructed using a Metagenomic DNA Library Kit (Matridx Biotechnology, China) and sequenced employing a NextSeq 550 High Output Reagent Cartridge ver. 2 (300 cycles; Illumina, USA).

Sequence analysis

Raw sequencing data were assessed for quality, trimmed, and then assembled *de novo* into a draft genome sequence using SPAdes ver. 3.14 software. The coverage of each sequence exceeded 98% and the sequencing depth was 100X. Multilocus Sequence Typing (MLST) was performed and the K serotypes, virulence scores, plasmid status, and antimicrobial resistance genes were determined as described on the Kleborate website (<https://>

[pathogen.watch/](#)). Kleborate is a new tool for evaluating hvKP virulence; it assigns a “virulence score” from 0 to 5. If the strain is negative for yersiniabactin, colibactin, and aerobactin, the score is 0; if it is yersiniabactin-positive only, 1; if it is yersiniabactin- and colibactin-positive (or colibactin-only positive), 2; if it is aerobactin-positive only, 3; if it is aerobactin- and yersiniabactin-positive and colibactin-negative, 4; and if it is yersiniabactin-, colibactin-, and aerobactin-positive, 5 (Lam et al., 2021).

Virulence genes were detected using the Virulent Factors of Pathogenic Bacteria (VFDB) database (http://www.mgc.ac.cn/VFs/search_VFs.htm). BioNumerics ver. 7.6 software was used to construct the minimum spanning tree. SKA was used to sort the sequencing results and obtain whole-genome single-nucleotide polymorphisms (SNPs). FastTree (version 2.1.11) software was employed for sequence alignment and homology analysis. The phylogenetic tree and heatmaps of the resistance and virulence genes were visualized using the ChiPlot tool (<https://www.chiplot.online/>). The genome sequences have been lodged with GenBank. The BioSample descriptor is SAMN40181878 and the accession numbers run from JBAOXF000000000 to JBAPAZ000000000.

Results

Isolate collection

During the 4-year screening period, 632 clinical isolates of KP from six sentinel hospitals were tested. Of these, 100 were positive in

the string test (15.8%) and were further analyzed. Most isolates were from respiratory specimens (n = 57, 57.0%), followed by urine (n = 23, 23.0%), blood culture (n = 14, 14.0%), and drainage (n = 6, 5.0%). The 100 isolates were collected from 100 patients, of whom 58 (58.0%) were male. Patient age ranged from 6 to 91 years.

Antimicrobial susceptibility profiles

The 100 string test-positive KP isolates were subjected to AST. High rates of resistance were observed for CTX (77.0%), SXT (67.0%), and NAL (64.0%); the rates for CT, TIG, and MEM were relatively low at 0.0%, 1.0%, and 3.0%, respectively. Some strains were resistant to 16 antibiotics (not CT). The resistance rates are shown in Table 1.

MLST and K-serotype

MLST identified 33 sequence types among the positive KPs, principally ST23, ST86, ST412, ST218, ST65, ST700, ST36, ST111, ST268, and ST1049. Of these, ST23 accounted for 16.0% (16/100), followed by ST86 (9.0%, 9/100) and ST412 (9.0%, 9/100). In all, 15 K serotypes were detected, with two strains having unknown serotypes. The dominant serotype was K1 (22.5% [22/98]), followed by K2 and K57 (20.4% [20/98] and 17.4% [17/98], respectively). The K serotypes were generally consistent within the same ST strains, thus ST23-K1, ST86-K2, ST412-K57, ST218-K57, and ST65-K2.

TABLE 1 Antimicrobial susceptibilities of string test-positive KP isolates.

Class	drug	susceptible		intermediate		Resistant	
		number	percentage (%)	number	percentage (%)	number	percentage (%)
β-lactam	ampicillin	1	1.0%	2	2.0%	97	97.0%
	ampicillin/sulbactam	62	62.0%	8	8.0%	30	30.0%
	ceftazidime	63	63.0%	0	0.0%	37	37.0%
	ceftazidime/avibactam	89	89.0%	2	2.0%	9	9.0%
	cefotaxime	23	23.0%	0	0.0%	77	77.0%
	ertapenem	94	94.0%	2	2.0%	4	4.0%
quinolones	meropenem	97	97.0%	0	0.0%	3	3.0%
	nalidixic acid	36	36.0%	0	0.0%	64	64.0%
	ciprofloxacin	75	75.0%	5	5.0%	20	20.0%
macrolides	azithromycin	86	86.0%	0	0.0%	14	14.0%
aminoglycosides	streptomycin	88	88.0%	4	4.0%	8	8.0%
	amikacin	89	89.0%	2	2.0%	9	9.0%
tetracyclines	tetracycline	82	82.0%	6	6.0%	12	12.0%
	tigecycline	99	99.0%	0	0.0%	1	1.0%
Phenylpropanols	chloramphenicol	31	31.0%	8	8.0%	61	61.0%

(Continued)

TABLE 1 Continued

Class	drug	susceptible		intermediate		Resistant	
		number	percentage (%)	number	percentage (%)	number	percentage (%)
sulfonamides	trimethoprim-sulfamethoxazole	33	33.0%	0	0.0%	67	67.0%
lipopeptides	colistin	100	100.0%	0	0.0%	0	0.0%

However, the two ST15 strains exhibited different K-serotypes, K19 and K24. The minimum spanning tree is shown in [Figure 1](#).

Antimicrobial-resistance genes

We identified 19 drug-resistance genes of 10 classes. The *SHV* point mutation, linked to penicillin resistance, was the most

common (98.0% of isolates); only two strains lacked *SHV* mutations. In the 98 strains with *SHV* variants, *SHV-11*, *SHV-1*, and *SHV-142* were the most prevalent. Eighty-five strains had single *SHV*-resistance genes but 15 had more than two genes. That are shown in [Figure 2](#). Resistance to penicillin mediated by *SHV*, *LAP-2*, *TEM-1D*, and *OXA-1* mutations was observed in 98.0%, 11.0%, 4.0%, and 1.0% of isolates, respectively. Genes mediating resistance to aminoglycosides, quinolones, carbapenems, sulfonamides, TET,

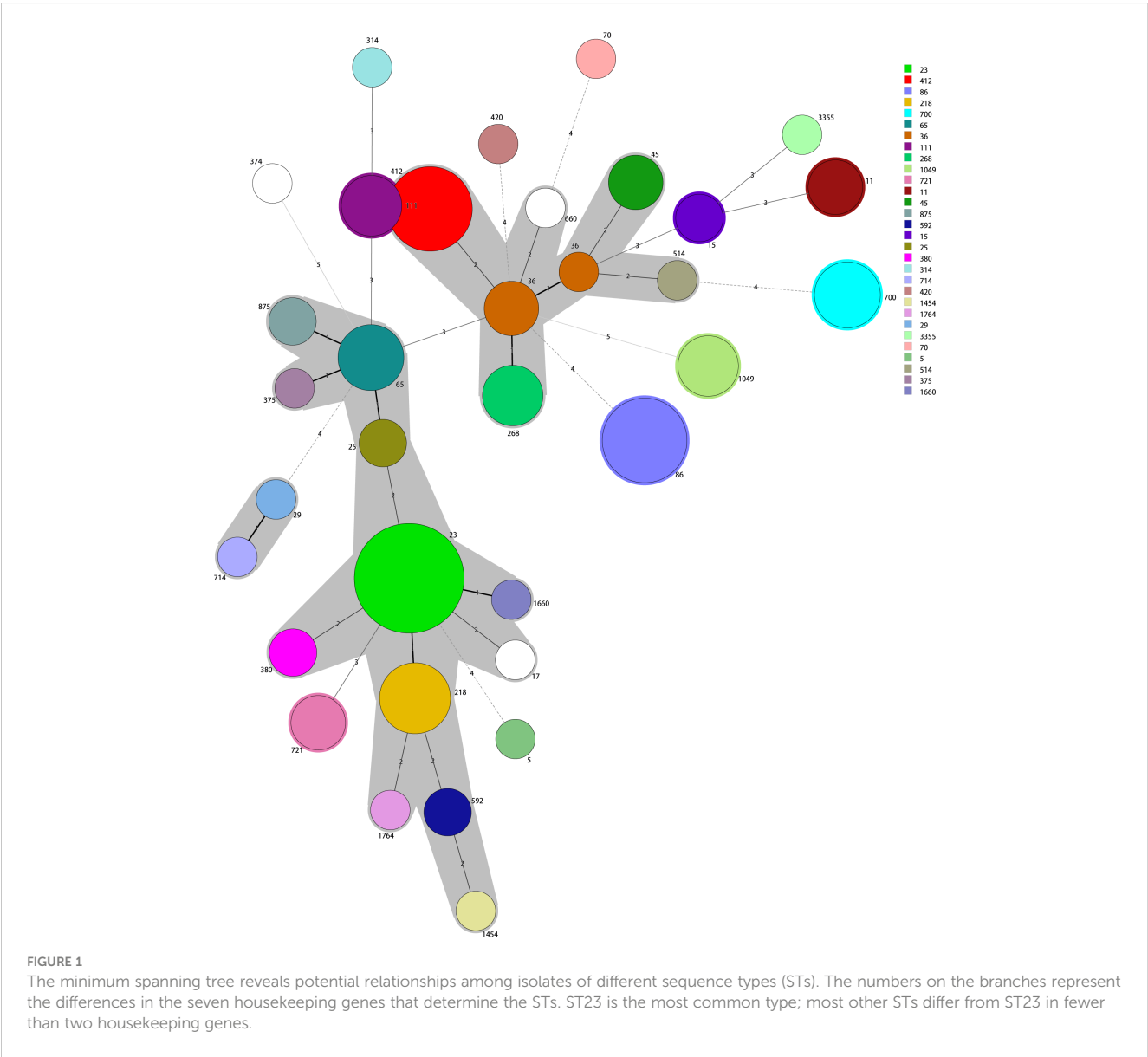


FIGURE 1
The minimum spanning tree reveals potential relationships among isolates of different sequence types (STs). The numbers on the branches represent the differences in the seven housekeeping genes that determine the STs. ST23 is the most common type; most other STs differ from ST23 in fewer than two housekeeping genes.

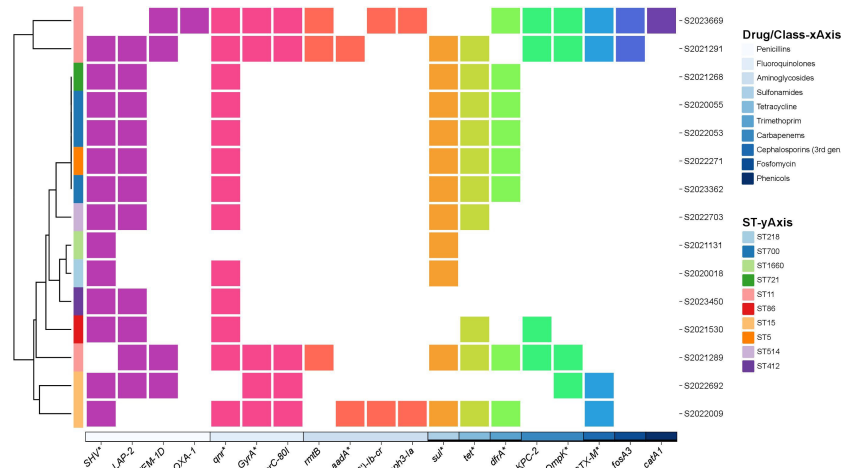


FIGURE 2
Annotation heatmap of antimicrobial resistance genes among 15 string test-positive KP strains with more than two resistance genes isolated in Huzhou from 2020 to 2023.

trimethoprim, cephalosporins (third generation), fosfomycin, and phenicol were identified. Antimicrobial resistance genes among 98 string test-positive KP strains are illustrated in [Supplementary Figure 1](#).

Virulence factors and scores

Virulence analysis using the VFDB database identified 112 virulence genes among the string test-positive KP isolates; Vfcass

divided these into seven groups based on their roles in pathogenesis. Adherence genes included type 3 fimbriae (*mrkA*, *mrkB*, *mrkC*, etc.), type I fimbriae (*fimA*, *fimB*, *fimC*, etc.), and type IV pili (*pilW*). Efflux pump genes included *acrAB* (*acrA*, *acrB*) and *farAB* (*farA*, *farB*). Iron uptake was affected by several virulence factors including mutations in aerobactin (*iucA*, *iucB*, *iucC*, etc.), the *ent* siderophore (*entA*, *entB*, *entC*, etc.), salmochelin (*IroB*, *IroC*, *IroD*, etc.), yersiniabactin (*fyuA*, *irp1*, *irp2*, etc.), and iron transport genes (*sitC* and *sitD*). Virulence factors affecting nutrition and regulation included mutations in genes of allantoin utilization

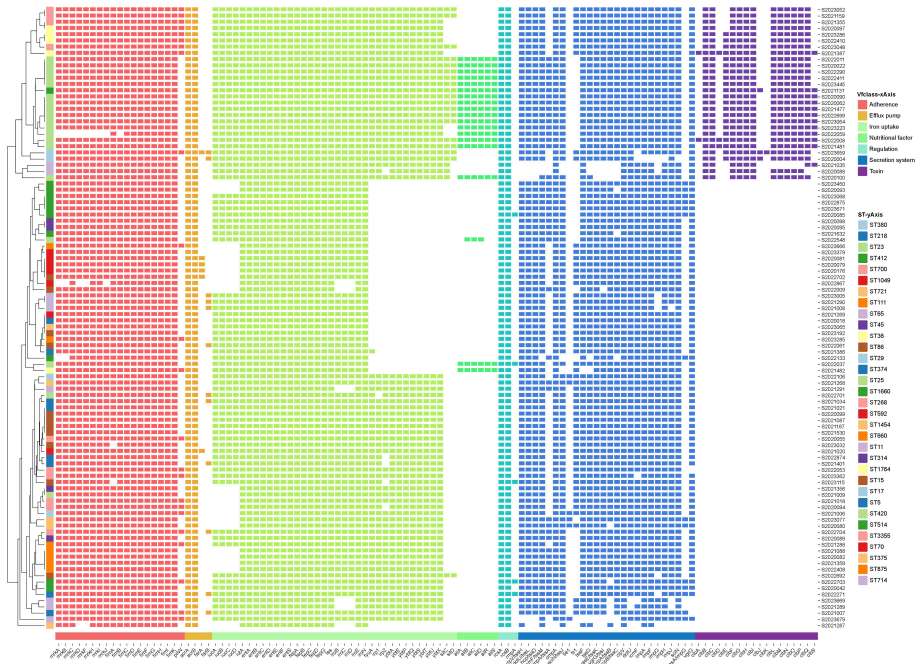


FIGURE 3
Annotation heatmap of virulence genes among 100 string test-positive KP strains in Huzhou from 2020 to 2023.

(*allA*, *allB*, *allC*, etc.) and *rscAB* (*rscA*, *rscB*, *rmpA*), respectively. The secretion system was affected by mutations in the T6SS-I (*ompA*, *tle1*, *tli1*, etc.) and T6SS-II/III (*dotU*, *icmF*, *impF*, etc.) clusters. The toxin genes included colibactin virulence factors (*clbA*, *clbB*, *clbC*, etc.). The virulence genes are illustrated in Figure 3.

The Kleborate virulence scores differed somewhat; not all 100 strains were hypervirulent. Twenty-eight strains scored 5, 28 scored 4, 21 scored 3, 12 scored 1, and 11 scored 0. The STs associated with virulence scores of 5 were ST23, ST380, ST268, ST36, and ST65.

Plasmids

No plasmids were detected in two strains but 24 plasmids (of seven families) were identified in the remaining 98 strains. The IncF plasmid was the most diverse (11 subtypes). Of the 24 plasmids, repB_KLEB (57.1%, 56/98), IncHI1B(pNDM-MAR)/repB_KLEB (28.6%, 28/98), and IncFIB(pKPHS1) (13.3%, 13/98) were the most common; the occurrence rates of other plasmids ranged from 1.0% to 10.2% (Figure 4).

Phylogenetic analysis

WgSNP phylogenetic analysis of the 100 string test-positive KP isolates identified three major clades designated A–C (Figure 5), but no association between any lineage and the year of isolation. Each clade included two subclusters (A1–A2, B1–B2, C1–C2). Clade A1 contained four strains (ST314, ST17, ST27, and ST714) and clade A2 had seven (ST1454, ST592, and ST111). Clades B1 and B2 contained 9 and 33 strains, respectively. The B1 ST types were ST70, ST25, ST375, and ST65. The B2 ST types were ST11, ST36, ST268, ST45, ST1764, ST218, ST721, and ST412. Clade C1 exhibited only one ST type, ST86 (nine strains). The largest clade, clade C2,

included 38 isolates with diverse ST types as follows: ST420, ST514, ST3355, ST660, ST5, ST15, ST374, ST380, ST700, ST875, ST1049, ST1660, and ST23.

Discussion

The latest data from the China Antimicrobial Resistance Surveillance System (CARSS) indicate that the KP clinical isolation rate is 21.2% of all Gram-negative bacteria, thus second to that of *E. coli*; KP is one of the most common causes of clinical infections in China. hvKP infections occur at unusual (sometimes multiple) sites, often accompanied by bacteremia and metastatic spread (Prokesch et al., 2016; Li et al., 2018; Wyres et al., 2020). To investigate the molecular epidemiological characteristics of hvKP in Huzhou, 100 KPs that were positive in string-tests were collected from 632 isolates, evaluated in terms of antimicrobial susceptibility, and subjected to WGS. Of these, 100 (15.82%) were string-test positive, fewer than the 32.03% in Guizhou province reported by Zhou et al. (2021) but more than the 7.63% in Germany reported by Neumann et al. (2023). The variation may be attributable to differences in economic or medical conditions. Respiratory specimens were the major source of positive isolates in Huzhou; patients with such infections require careful attention.

Antimicrobial resistance has long compromised human health. The misuse of antibiotics in clinical practice, agriculture, and aquaculture has exacerbated the problem (Arias and Murray, 2009). hvKP antimicrobial resistance can arise via acquisition of a plasmid carrying resistance genes, chromosomal gene mutations, or the transfer of hypervirulent plasmids to multidrug-resistant strains (Dong et al., 2018; Feng et al., 2018; Gu et al., 2018). All hvKP strains exhibit an inherent resistance to AMP; we observed a high resistance rate (Russo and Marr, 2019). Of the 97 AMP-resistant strains, 100.0% (n = 97) harbored the *SHV* gene. High resistance

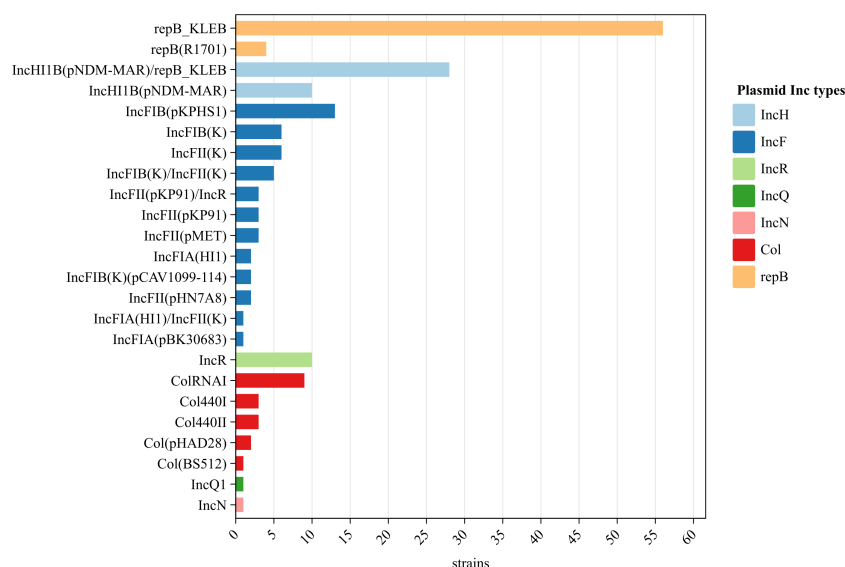


FIGURE 4
Plasmids of the 100 string test-positive KP strains in Huzhou from 2020 to 2023.

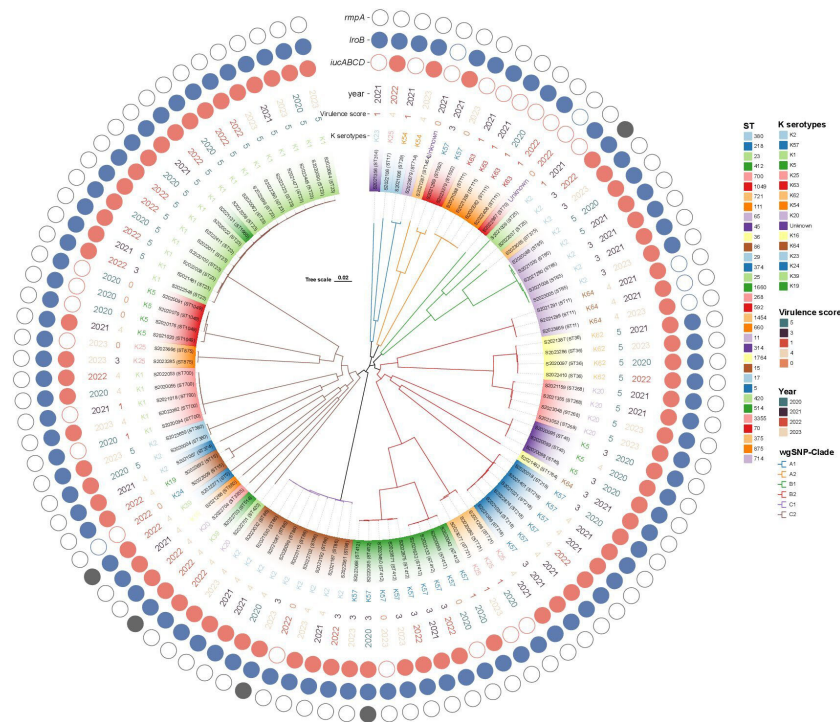


FIGURE 5

Phylogenetic analysis of 100 string test-positive KP isolates in Huzhou from 2020 to 2023.

rates were also observed for NAL, CHL, and SXT, consistent with previous findings (Wang et al., 2022). Caution should be exercised when using these antibiotics to treat hvKP infections. Notably, we found no CT-resistant strain. CT is very active against most Gram-negative bacteria and is often considered the “last resort” treatment for multidrug-resistant bacteria (Biswas et al., 2012).

Genomic studies have shown that KP (including hvKP) genomes are diverse but highly structured (Wyres et al., 2020). Our 100 isolates exhibited the hypermucoviscous phenotype (positive string test results) and were of 33 ST types with 15 capsular serotypes. Notably, the K1 capsular type was the predominant serogroup responsible for KP infections in Huzhou; most K1-type strains were of the ST23 type, as reported by Tang et al. (2020) and Hallal Ferreira Raro et al. (2023). The most common capsular locus associated with positive KPs was K1 (22.5%), followed by K2 (20.4%), K57 (17.4%), and K5 (7.1%), consistent with other regional studies (Lee et al., 2016; Guo et al., 2017). Most positive strains of the K1 serotype were of type ST23, but K2 serotype strains were of several ST types including ST86, ST65, and ST25, as in previous studies (Struve et al., 2015; Anantharajah et al., 2022). In addition, we report a K20 ST3355 isolate. New hvKP STs continue to emerge, indicating ongoing evolution (Lam et al., 2018; Wyres et al., 2020). The minimum spanning tree divided the 100 strains into two evolutionary branches with ST23 and ST412 as the cores.

hvKP is inherently resistant to AMP because a β -lactamase enzyme is encoded by chromosomal *SHV*. Of our strains, 98 with *SHV* genes exhibited wild-type *in vitro* susceptibility to β -lactams. Of these, 85 carried only one *SHV* resistance gene, but the

remaining 15 had more than two, including genes conferring resistance to various antibiotics such as penicillin (*LAP-2*, *TEM-1D*, *OXA-1*), aminoglycosides (*rmlB*, *aadA*, *aac(6')-Ib-cr* and *aph3-Ia*), fluoroquinolones (*qnr*, *GyrA*, and *ParC-80I*), carbapenems (*OmpK* and *KPC-2*), sulfonamides (*sul*), TET (*tet*), trimethoprim (*dfrA*), cephalosporins (*CTX-M*), fosfomycin (*fosA3*), and phenicols (*catA1*). Specific mutations in the outer membrane porin *OmpK* gene contribute substantially to the carbapenem-resistance of hvKP. Porin defects can increase the MIC to above the levels conferred by acquired carbapenemase genes alone (Fajardo-Lubián et al., 2019). The *fosA3* gene of hvKP reduces fosfomycin susceptibility to below the breakpoint of clinically relevant resistance. ESBLs (encoded by *CTX-M*, *SHV*, and *TEM*, and so forth) are modified broad-spectrum β -lactamases that hydrolyze third-generation cephalosporins, aztreonam, and fourth-generation cephalosporins. hvKPs that express ESBLs may also possess porin mutations that decrease the uptake of cephalosporins and carbapenems, further reducing susceptibility to such agents (Surgers et al., 2016; Yu et al., 2017). Of the four strains exhibiting ETP-resistant phenotypes, all carried the *KPC-2* carbapenem-resistance gene. *KPC* transposon-mediated spread is increasingly reported globally. Such spread is frequently coupled to the dissemination of other β -lactamases (Xu et al., 2019); here, we describe the coexistence of a *KPC* gene and *ESBL*-encoding genes.

Although many studies have defined hvKP as a positive result in the string test, not all hvKP strains are hypermucoviscous (Fu et al., 2018; Russo and Marr, 2019). The predominant aerobactin siderophore enhances virulence, and it is likely to be a reliable biomarker of hvKP strains (Russo et al., 2015). Russo et al. (2018)

reported that *iucA* of the *iuc* operon encoding aerobactin very accurately differentiated hvKP from cKP strains; *iucA*-positivity indicated hypervirulence. As shown in Figures 3, 5, 78.0% (78/100) of strains carried *iucA*; KP hypervirulence can thus be preliminarily indicated by a positive string test. The status of *iroB* (encoding an enzyme of salmochelin siderophore biosynthesis) is also important when determining whether a strain is hypervirulent (Russo et al., 2018); the carrying rate of *iroB* was 94.0% (94/100) in our study. The biosynthesis of salmochelin and aerobactin is encoded by the *iroB* and *iucA* clusters of virulence plasmids, respectively. *RmpA* is a plasmid-located virulence factor that regulates the synthesis of capsular polysaccharides (Wang et al., 2020). Lin et al. (2020) found that *rmpA* deletion eliminated the hypermucoviscous phenotype and decreased virulence; *rmpA* overexpression increased virulence. Although *rmpA* has been considered a useful genetic marker of hvKP, only five of our strains carried it. Thus, although *rmpA* increases virulence, it may not be the optimal indicator of hypervirulence (Neumann et al., 2023).

Not all KP strains that are positive in string tests exhibit high virulence scores (Russo et al., 2018); 11 of our strains had virulence scores of 0 and were thus hypermucoviscous cKP (non-hypervirulent) strains. As shown in Figure 5, virulence was not closely associated with the ST type or serotype but rather with *iucA* gene-positivity. The *iucA* gene was not detected in 11 strains, but of these, 8 (72.7%) carried the *iroB* gene. Of the 28 strains that scored 5 in the virulence test, ST23-K1 accounted for 53.6% (15/28), ST36-K62 for 14.3% (4/28), and ST268-K20 for 14.3% (4/28). Interestingly, patient age was associated with virulence scores; 78.6% ($n = 22$) of strains with virulence scores of 5 ($n = 28$) were from patients over 60 years of age, perhaps reflecting poor immune function in the elderly. The *iucA* gene was detected in all 77 strains with virulence scores of 3, 4, and 5. Thus, *iucA*-positivity well-reflects hypervirulence status.

The widespread IncF plasmid of Enterobacteriaceae typically carries β -lactamase and carbapenemase genes that significantly contribute to KP drug resistance. The IncR plasmid is predominantly found in *Salmonella* and can harbor various drug-resistance genes associated with multiple drug-resistance phenotypes in human hosts. This plasmid can be horizontally transferred between bacterial strains (Qian et al., 2020).

String test-positive KP strains in Huzhou have been understudied. We used wgSNP phylogenetic analysis to show that strains from patients in Huzhou could be categorized into six main clades (A1, A2, B1, B2, C1, and C2). Strains sharing the same STs were more closely related and tended to carry the same virulence and antibiotic-resistance genes. Interestingly, closely related strains were found in both the same and different hospitals, and in the same and different years. Associations between clades and certain resistance and virulence determinants were common, although not all clades of a phylogenetic tree exhibited particular geographic distributions or years of isolation.

Physicians who encounter invasive infections caused by KP should prioritize prompt microbiological tests that differentiate hvKP from cKP via molecular characterization of the major virulence factors. Virulence genes commonly found in hvKP strains (e.g., *iucA*) might serve as genotypic biomarkers for early detection of hvKP in areas of low KP prevalence. Today, hvKP poses a significant

public health threat; rigorous monitoring is essential to prevent widespread transmission and potential infectious outbreaks.

Our work had several limitations. First, a positive result in the string test does not inevitably indicate hypervirulence. Catalán-Nájera et al. (2017) noted that not all hvKPs exhibit a hypermucoviscous phenotype; thus, we may have missed some hvKPs during screening. Second, we lacked details on the infections and treatment results; we do not know what harm the patients suffered.

Conclusions

We present an extensive genomic analysis of KPs isolated in Huzhou that tested positive in the string test. This affords valuable insights into ST prevalences, capsular serotypes, antimicrobial-resistance markers, virulence genes, and phylogenetic relationships from 2020 to 2023. The rates of resistance to CTX, SXT, and NAL were high but resistance to CT was low. The *iucA* gene of hvKP may serve as a genotypic biomarker for early detection. It is essential to develop monitoring, control, and prevention strategies that address the increasing threats posed by hvKP.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by the human research ethics committee of the Huzhou Center for Disease Control and Prevention. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

WY: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. DX: Investigation, Methodology, Writing – review & editing. YS: Validation, Writing – original draft. FD: Software, Writing – original draft. LJ: Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1411658/full#supplementary-material>

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Clinical outcomes and risk factors of *Acinetobacter baumannii* meningitis in pediatric patients at a tertiary hospital in China

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Objectives: To summarize the clinical characteristics, outcomes and identify risk factors of *Acinetobacter baumannii* (AB) meningitis in children.

Methods: This was a single-center, retrospective study. Children hospitalized between January 2016 and December 2021 who were diagnosed with AB meningitis were included. The clinical characteristics and outcomes were reviewed. Risk factors were determined using univariate analyses (chi-square and Mann-Whitney U tests).

Results: Seventeen patients were included; 15 cases were secondary to neurosurgery, and two were neonates with primary bacterial meningitis. Common symptoms included fever, convulsions and nervous system abnormalities. Cerebrospinal fluid (CSF) tests typically showed increased white blood cell counts dominated by neutrophils, reduced glucose levels and elevated protein levels. Ten patients were successfully treated (successful treatment [ST] group); seven had failed treatment (failed treatment [FT] group). Univariate analyses revealed that mechanical ventilation, routine white cell counts in the peripheral blood, procalcitonin, protein in the CSF, septic shock and carbapenem-resistant AB (CRAB) differed significantly between the groups.

Conclusion: AB meningitis in children has a high mortality rate. FT was associated with mechanical ventilation, septic shock, CRAB, lower peripheral leukocyte counts, higher protein levels in the CSF and procalcitonin. Larger studies are needed to identify independent risk factors for adverse outcomes.

KEYWORDS

Acinetobacter baumannii, meningitis, carbapenem-resistant, clinical characteristics, outcomes, children

Introduction

Acinetobacter baumannii (AB), a gram-negative coccobacillus, is an opportunistic pathogen. It is primarily associated with patients admitted to hospitals or undergoing healthcare-related treatments, particularly those who receive treatment in intensive care units (ICUs). AB can cause multiple system infections, including pneumonia, bloodstream infections, meningitis, skin infections and soft tissue infections.

Bacterial meningitis is among the most difficult diseases to treat in children and exhibits a poor prognosis. AB is a significant nosocomial meningitis agent (Chusri et al., 2018) and accounts for >25% of all pathogens isolated from cerebrospinal fluid (CSF) (Chen et al., 2021). However, the resistance rates of AB to various antibiotics continue to increase yearly. Few antibacterial drugs are available to treat such bacterial infections, presenting great therapeutic challenges (Tamma et al., 2022). The current treatments are mainly combining at least two antibiotics based on tigecycline, polymyxin B, sulbactam or carbapenems (Tamma et al., 2023). Bacterial meningitis with extensively drug-resistant pathogens can lead to ineffective antibacterial treatment and has the highest mortality rates among infections (Mauldin et al., 2010). A previous study reported that AB-induced central nervous system (CNS) infections account for a high risk of death in pediatric patients (Shi et al., 2020). AB meningitis is rare in children. The lack of clinical cases, nonspecific clinical manifestations, limited drugs choices, and poor immunity in children increase the severity of the damage caused by this infection and the challenges of clinical treatment (Ye et al., 2020).

AB meningitis often occurs secondary to neurosurgery, leading to high mortality rates and neurological sequela (Chusri et al., 2018). Most previous studies have focused on the risk factors for the occurrence of AB meningitis, such as neurosurgery, head trauma, CSF leakage, wound infection, and foreign body implantation (Korinek et al., 2006). However, few studies have focused on the risk factors for poor outcomes.

Carbapenem-resistant AB (CRAB) has become increasingly problematic in recent years (Kyriakidis et al., 2021). A previous systematic review reported that CRAB is a leading pathogen associated with deaths attributable to bacterial antimicrobial resistance and caused more than 50000 deaths in 2019. Furthermore, CRAB was the fourth leading pathogen-drug combination globally for 2019 (Antimicrobial Resistance, 2022). Patients with CRAB have double the mortality rate and a significantly greater burden of illness compared with those with carbapenem-susceptible AB (CSAB) (Lemos et al., 2014; Pogue et al., 2022). Multiple studies have shown that CRAB was the most common pathogen in non-survivors of bacterial meningitis, and the worst clinical outcomes often occurred with CRAB infections (Shi et al., 2020; Thatrimontrichai et al., 2021; Panic et al., 2022; Zheng et al., 2022). However, few studies have compared the differences in clinical manifestations and outcomes between meningitis patients with CRAB vs CSAB.

Few reports have been published on AB meningitis in children, and of these, few were case reports or reviews. Therefore, we

retrospectively analyzed children with this infection who were hospitalized at our institution. Our primary objective was to summarize the clinical characteristics and outcomes and identify key factors associated with the infection, thereby enhancing clinicians' understanding and management of this serious condition.

Methods

Setting and study design

This retrospective study was conducted in the Children's Hospital of Fudan University, a national children's medical center. Children (aged ≤18 years) hospitalized from 1st January 2016 to 31st December 2021 from whom AB was detected in the CSF were included. Only the first episodes of positive cultures was included to avoid case duplication.

The inclusion criteria were (1) isolation of AB from the CSF; (2) increased white blood cell counts ($\geq 10 \times 10^6/L$) and protein levels (≥ 450 mg/L) and decreased glucose in the CSF; and (3) clinical evidence of bacterial meningitis (i.e., fever, headache, vomiting, confusion, irritability and meningeal irritation). The exclusion criteria were (1) incomplete medical records and (2) the cases in which positive CSF cultures were obtained in the absence of clinical and laboratory features of meningitis.

Patients were divided into either the successful treatment (ST) or failed treatment (FT) group according to patient outcomes. ST was defined as two consecutive negative CSF cultures with clinical symptoms that had disappeared and normal routine peripheral blood and CSF tests. FT was defined as persistent positive CSF cultures before discharge or patient's eventual death.

Patients were also divided into CRAB group and CSAB group according to results of susceptibility testing. And we compared clinical characteristics between the two groups.

Data collection

Medical records of all included patients were reviewed. Data extracted included demographic information, hospital stay length, hospitalization ward, days from first neurosurgery to diagnosis, clinical features, diagnosis, underlying diseases, previous hospital admission within 6 months, invasive procedures, and receipt of immunosuppressive therapy (including cytotoxic agents within 6 weeks or corticosteroids at a dosage ≥ 10 mg of prednisolone daily for >5 days within 4 weeks prior to detection (Chusri et al., 2019). Additional data included antibiotic resistance, previous antibiotic therapy within 30 days, co-infections with other bacteria or fungi, antibiotic treatment, time to CSF sterilization, and patient outcomes. Sepsis and septic shock were evaluated by clinician according to the Sepsis-3 (Singer et al., 2016). And neonatal sepsis was evaluated by clinician according to the consensus of domestic experts (Subspecialty Group of Neonatology, t. S. o. P. C. M. A and Professional Committee of Infectious Diseases, N. S. C. M. D. A, 2019).

Antibiotic resistance and antimicrobial susceptibility testing

We reviewed the antibiotic-resistance profiles of included AB isolates. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics, Bremen, Germany) was used to confirm the species of the isolates. AST was performed in the microbiology laboratory of the hospital using the Kirby-Bauer method or automated systems (VITEK 2 Compact). AST was conducted for 14 agents: imipenem, meropenem, levofloxacin, amikacin, gentamicin, ceftazidime, cefepime, piperacillin-tazobactam, ampicillin-sulbactam, cefoperazone-sulbactam, colistin, minocycline, tigecycline and sulfamethoxazole-trimethoprim (TMP-SMX). Antibiotic susceptibilities were defined according to the criteria of the [Clinical and Laboratory Standards Institute, 2022](#) breakpoints ([Clinical and Laboratory Standards Institute, 2022](#)). CRAB was defined as having minimum inhibitory concentrations (MICs) of imipenem or meropenem ≥ 8 mg/L. CSAB was defined as having MICs of imipenem and meropenem ≤ 2 mg/L.

Statistical analyses

Categorical data were summarized as counts and percentages; chi-square and Fisher's exact tests were used for comparisons between two groups. Continuous data were presented as means $\pm$ standard deviation or median with interquartile range (IQR), depending on the degree of skewness in the distributions evaluated using the Shapiro-Wilk test. Differences were identified using t-tests or Mann-Whitney U tests. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS statistical software, version 23.0.

Results

Patients' clinical characteristics

Seventeen patients (9 boys; 8 girls) with AB meningitis hospitalized from 1st January 2016 to 31st December 2021 were included. [Table 1](#) showed their clinical characteristics. The median age of the patients was 24.00 months (IQR, 17.00–107.00 months); only one patient was a neonate. Ten patients (58.82%) were in the ICU; seven (41.18%) were in general wards. Of the 17 patients, 15 were cases secondary to neurosurgery; two were neonates with primary bacterial meningitis. The average time from the first surgery to positive CSF culture was 15.00 days (IQR, 4.50–24.00 days). All patients had underlying diseases, including craniocerebral tumor (12% [70.59%]), intracranial hemorrhage (1 [5.88%]), hydrocephalus (2 [11.76%]) and neonatal sepsis (2 [11.76%]).

All patients had clinical manifestations of recurrent fever and disturbance of consciousness. 11 patients had vomiting; five had convulsions, and three were positive for meningeal irritation.

During treatment, five patients developed complications: four had hydrocephalus, and one had subdural effusion. Routine peripheral blood tests showed increased white blood cell counts dominated by neutrophils, c-reactive protein (CRP) levels averaging 87.80 ± 51.23 mg/L and increased procalcitonin (PCT) with an average of 1.03 (IQR, 0.11–2.92) ng/mL. The CSF changed typically: increased white blood cell counts with an average of $1160.00 \times 10^6/L$ (IQR, 495.00–2485.00) dominated by neutrophils; decreased glucose with an average of 0.80 mmol/L (IQR, 0.05–1.85), and increased protein contents averaging 3577.90 mg/L (IQR, 2134.10–5755.00). Six patients (35.29%) had sepsis; seven (41.18%) developed septic shock.

Twelve patients had CRAB; five had CSAB according to antibiotic resistance. We compared clinical characteristics between them. Hospitalization ward ($P = 0.003$) and distribution of underlying conditions ($P = 0.010$) differed significantly between them. Among CRAB patients, 11 (91.67%) had craniocerebral tumors, whereas only one CSAB patient (20.00%) had a craniocerebral tumor. Protein in the CSF and the proportion of septic shock were significantly higher in the CRAB group than in the CSAB group. Conversely, the proportion of CSF sterilization was significantly lower in the CRAB group than in the CSAB group.

AST

[Figure 1](#) showed the AST results of the AB isolates to common clinical antibiotics. Of these, 70.6% of the strains were resistant to meropenem and imipenem. The resistance rates to ceftazidime, cefepime, piperacillin-tazobactam, ampicillin-sulbactam, cefoperazone-sulbactam, amikacin, gentamicin and TMP-SMX were 70.6%, 70.6%, 70.6%, 64.7%, 64.7%, 64.7%, 64.7% and 52.9%, respectively. The resistance rates were lower to levofloxacin (29.4%) and minocycline (5.9%) than to other antibiotics, whereas the intermediate rates were higher (41.2% and 35.3%, respectively). No isolates that were resistant to colistin or tigecycline.

Treatment and outcomes

Two patients had neonatal sepsis. Four patients received antibiotics to prevent perioperative infection and 11 cases underwent anti-infective treatment before being diagnosed with meningitis. When the CSF culture was positive, the treatment plan was actively adjusted according to antibiotic susceptibility. Patients with CSAB were treated primarily with dual antibiotic therapy based on meropenem, and other antibiotics included ampicillin-sulbactam, cefoperazone-sulbactam, amikacin, and fosfomycin. Of the patients with CRAB, three received dual antibiotic therapy, including meropenem and ampicillin-sulbactam, levofloxacin and sulbactam sodium, ampicillin-sulbactam and polymyxin B. Seven received triple antibiotic therapy based on meropenem and tigecycline; other antibiotics included fosfomycin, quinolones and sulbactam. The remaining two patients eventually received quadruple antibiotic

TABLE 1 Characteristics of patients with *Acinetobacter baumannii* meningitis.

Characteristics	All patients (N=17)	CRAB (N=12)	CSAB (N=5)	P-value
Male	9(52.94%)	6(50.00%)	3(60.00%)	1.000
Age, m, median (IQR)	24.00(17.00,107.00)	23.50(17.00,142.75)	28.00(9.49,50.00)	0.598
Weight, kg, median (IQR)	16.88 ± 12.51	19.01 ± 14.01	11.78 ± 6.38	0.165
Length of hospital stay, d, median (IQR)	91.65 ± 62.10	102.42 ± 67.82	65.80 ± 39.96	0.191
Hospitalization ward				
Pediatric intensive care unit	10(58.82%)	10(83.33%)	0(0.00%)	0.003
General wards	7(41.18%)	2(16.67%)	5(100.00%)	
Clinical features of infections				
Duration of fever, d, median (IQR)	20.12 ± 10.51	22.25 ± 11.37	15.00 ± 6.28	0.204
White cell count, ×10 ⁹ /L, median (IQR)	17.00(12.95,24.75)	16.00(12.55,28.70)	17.00(12.65,20.90)	0.527
Neutrophil, %, median (IQR)	76.70(61.05,83.00)	81.05(62.70,83.55)	68.90(60.85,75.15)	0.154
C-reactive protein, mg/L, median (IQR)	87.80 ± 51.23	78.22 ± 53.40	110.80 ± 41.46	0.244
Procalcitonin, ng/ml, median (IQR)	1.03(0.11,2.92)	1.13(0.19,3.30)	0.14(0.06,2.18)	0.141
Interleukin 6, pg/ml, median (IQR)	126.60(41.78,262.60)	235.70(126.60,283.90)	45.02(38.55,46.30)	0.087
Sepsis	6(35.29%)	5(41.67%)	1(20.00%)	0.600
Septic shock	7(41.18%)	7(58.33%)	0(0.00%)	0.044
White cell count in CSF,×10 ⁶ /L, median (IQR)	1160.00(495.00,2485.00)	1200.00(625.00,2492.50)	1000.00(170.00,1830.00)	0.205
Protein in CSF, mg/L, median (IQR)	3577.90 (2134.10,5755.00)	4744.20 (3236.73,7500.90)	2306.50 (1134.30,2643.45)	0.011
Glucose in CSF, mmol/L, median (IQR)	0.80(0.05,1.85)	0.10(0.00,1.50)	1.60(1.15,2.35)	0.071
Underlying condition				0.010
Craniocerebral tumor	12(70.59%)	11(91.67%)	1(20.00%)	
Intracranial hemorrhage	1(5.88%)	0(0.00%)	1(20.00%)	
Hydrocephalus	2(11.76%)	0(0.00%)	2(40.00%)	
Neonatal sepsis	2(11.76%)	1(8.33%)	1(20.00%)	
Neurosurgery	15(88.24%)	11(91.67%)	4(80.00%)	0.515
Days from first neurosurgery to diagnosis, d, median (IQR)	15.00(4.50,24.00)	10.50(4.25,24.00)	20.00(7.50,31.50)	0.527
Duration of targeted antibiotic treatment, d, median (IQR)	30.00(18.50,42.00)	34.00(14.75,42.00)	23.00(20.00,34.00)	0.597
CSF sterilization				
Yes	10(58.82%)	5(41.67%)	5(100.00%)	0.044
No	7(41.18%)	7(58.33%)	0(0.00%)	

CSF, cerebrospinal fluid; CRAB, carbapenem-resistant *Acinetobacter baumannii*; CSAB, carbapenem-susceptible *Acinetobacter baumannii*.

therapy (meropenem, sulbactam sodium, polymyxin B and levofloxacin/fosfomycin). Three patients received intrathecal injections of polymyxin B. The average time for targeted antibiotic treatment was 30.00 days (IQR, 18.50–42.00), lasting 2–4 weeks after two consecutive negative CSF cultures. Finally, ten patients’ CSF cultures became negative. Seven patients died; all of whom had CRAB, and their CSF cultures remained positive.

Univariate analysis of patients with AB meningitis, stratified by outcome

Of the seventeen patients included in the final analysis, 10 had ST, and seven had FT. Univariate analyses revealed that mechanical ventilation ($P=0.044$), routine white blood cell counts in the peripheral blood ($P=0.031$), PCT ($P=0.045$), protein in the CSF

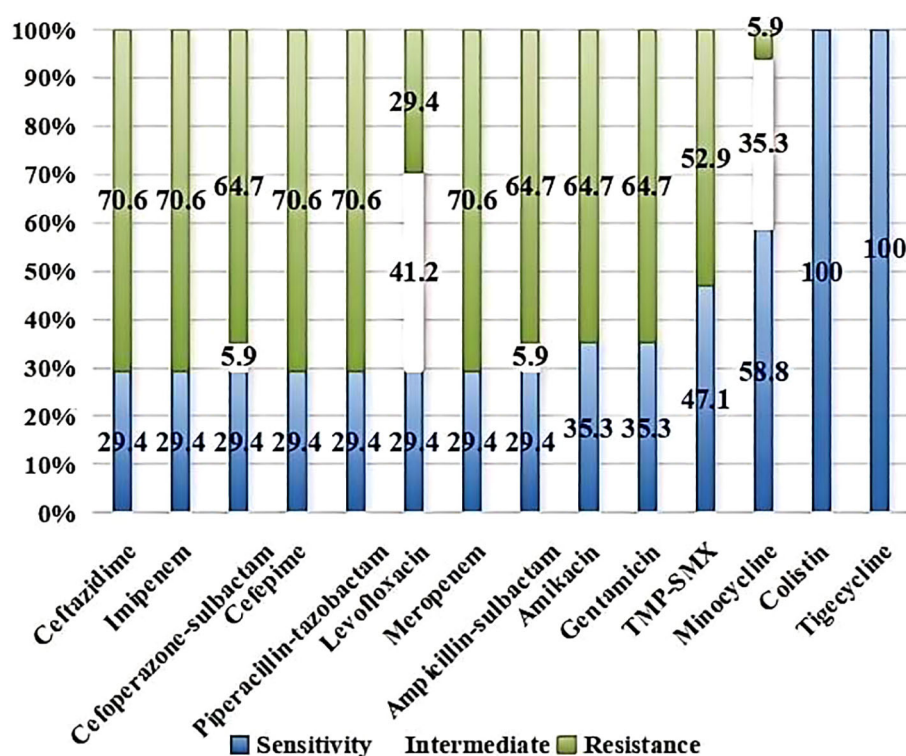


FIGURE 1
AST of *Acinetobacter baumannii*.

($P=0.032$), septic shock ($P=0.004$) and CRAB ($P=0.044$) differed significantly between the groups (Table 2).

Discussion

AB has become a major problematic pathogen in healthcare settings for extensive drug resistance. All-cause mortality from *Acinetobacter* spp. meningitis is reported to range from 15%–71%, and the highest mortality rates have been observed in neonates (Kim et al., 2009). Children have weaker immunity; thus, when infection occurs, the harm is more serious. Therefore, special attention should be paid to AB meningitis.

AB meningitis is mostly secondary to neurosurgery (Xiao et al., 2019). In our study, 15/17 patients (88.24%) underwent neurosurgery. Owing to a lack of specificity of clinical manifestations, clinicians often have difficulty identifying AB meningitis in time (Saleem et al., 2011). Most patients presented with fever, convulsions, and nervous system abnormalities. Blood routine examinations showed that inflammatory indicators, including CRP, PCT and interleukin-6, were significantly increased. CSF tests typically showed increased white blood cell counts dominated by neutrophils, reduced glucose levels and significantly elevated protein levels.

Because the safety of children's medication is crucial, the choice of antibiotics is greatly limited. The guidance by Infectious Diseases Society of America (IDSA) recommended combining at least two active antibiotics when possible to treat AB infection (Tamma et al.,

2023). For first-line treatment, meropenem is the most commonly recommended empiric treatment (Tunkel et al., 2017). In our study, patients with CSAB were treated mainly with meropenem. However, its resistance rate exceeds 70%. In addition, CRAB strains were resistant to most antibiotics except polymyxins and tigecycline. For these strains, guidelines recommend administering polymyxin B intravenously or in combination with intrathecal injection to treat CRAB meningitis (Tunkel et al., 2017; Chinese Research Hospital Association Of Critical Care, M et al., 2019). Previous literature showed for extensively drug-resistant AB meningitis, patients treated with intravenous and intrathecal/intracerebral ventricle injection of polymyxin B had a significantly lower 28-day mortality (55.26% vs. 8.70%, $P=0.01$) and higher rates microbiological clearance (91.30% vs. 18.42%, $P<0.001$) compared with patients treated with other antibiotics (Pan et al., 2018). In our study, two patients received intrathecal injections of polymyxin B and one patient received intravenous polymyxin B in combination with intrathecal injection. They were all treated successfully. Although CRAB is usually resistant to carbapenems, clinicians still try to combine carbapenems with other drugs to treat CRAB infections clinically. Studies have shown that high-dose, prolonged infusions of meropenem combined with other sensitive antibiotics have synergistic effects (Yu et al., 2018; Zhou et al., 2021). Therefore, among twelve patients with CRAB infections, all but two were treated with meropenem and eight cases received high-dose, prolonged infusions. However, this approach did not have a favorable outcome. Only five patients treated with meropenem were eventually cured. Among them, three cases were also treated

TABLE 2 Univariate analysis of factors associated with the FT group compared with those of the ST group.

Characteristic	FT N=7	ST N=10	P value
Age, m, median (IQR)	70.00 (20.00,146.00)	22.00 (12.26,39.00)	0.143
Male sex	3(42.86%)	6(60%)	0.637
Duration of hospital stay before detected, d, median (IQR)	27.00 (12.00,53.00)	19.00(6.75,30.00)	0.329
Previous hospital admission within 6 m	2(28.57%)	7(70.00%)	0.153
Neurosurgery	7(100.00%)	8(80.00%)	0.485
Days from first neurosurgery to diagnosis, d, median (IQR)	9.00(5.00,25.00)	17.50(2.25,24.00)	0.845
Mechanical ventilation	7(100.00%)	5(50.00%)	0.044
Indwelling central venous catheter	6(85.71%)	5(50.00%)	0.304
Indwelling ventricular drainage	4(57.15%)	3(30.00%)	0.644
Receiving immunosuppressive therapy	1(14.29%)	0(0.00%)	0.412
Co-infections with other bacteria or fungi	2(28.57%)	4(40.00%)	1.000
Intrathecal injection	0(0.00%)	3(30.00%)	0.228
Previous antibiotic therapy within 30 d			
Cephalosporins	7(100.00%)	8(80.00%)	0.485
β-Lactam-β-Lactamase Inhibitor	4(57.15%)	2(20.00%)	0.162
Carbapenems	6(85.71%)	5(50.00%)	0.304
Vancomycin	4(57.15%)	7(70.00%)	0.644
Clinical features of infections			
Duration of fever, d, median (IQR)	19.90 ± 10.01	20.43 ± 12.00	0.923
White cell count, ×10 ⁹ /L, median (IQR)	14.85 ± 4.45	25.34 ± 9.88	0.031
Neutrophil, %, median (IQR)	82.30 (76.70,86.10)	69.70 (58.60,80.70)	0.107
C-reactive protein, mg/L, median (IQR)	76.87 ± 48.31	103.43 ± 54.90	0.308
Procalcitonin, ng/ml, median (IQR)	1.87(1.07,4.36)	0.14(0.90,2.18)	0.045
Interleukin 6, pg/ml, median (IQR)	47.58 (38.55,186.10)	47.58 (32.07,235.70)	0.210
White cell count in CSF, ×10 ⁶ /L, median (IQR)	1200.00 (1000.00,2470.00)	1080.00 (198.75,2500.00)	0.463
Protein in CSF, mg/L, median (IQR)	4960.00 (3577.90,7980.20)	2440.25 (1415.48,4541.00)	0.032

(Continued)

TABLE 2 Continued

Characteristic	FT N=7	ST N=10	P value
Clinical features of infections			
Glucose in CSF, mmol/L, median (IQR)	0.10(0.00,1.70)	1.20(0.18,2.15)	0.184
Sepsis	5(71.43%)	2(20.00%)	0.058
Septic shock	6(85.71%)	1(10.00%)	0.004
Hospitalization ward			0.134
Pediatric intensive care unit	6(85.71%)	4(40.00%)	
General wards	1(14.29%)	6(60.00%)	
Detection with CRAB	7(100.00%)	5(50.00%)	0.044
Duration of targeted antibiotic treatment, d, median (IQR)	17.00 (13.00,42.00)	34.00 (22.25,42.00)	0.186

ST, successful treatment group; FT, failed treatment group; CRAB, carbapenem-resistant *Acinetobacter baumannii*; CSF, cerebrospinal fluid.

with intrathecal injection of polymyxin B, and two cases were treated with ampicillin-sulbactam. Therefore, successful treatment did not seem to be attributable to meropenem alone. Tigecycline is another first-line antibiotic for treating CRAB; however, its concentration in the CSF is limited (Rodvold et al., 2006). Therefore, tigecycline has limited role in CRAB meningitis (Ye et al., 2020). Seven of our patients received therapy with meropenem and tigecycline, and other antibiotics included fosfomycin, quinolones or sulbactam. However, treatment ultimately failed in six of them. Therefore, clinicians must better understand CRAB treatment, and rational selection of antibacterial drugs is crucial for treating meningitis. Additionally, sulbactam is also an important option for the treatment of CRAB infections. The guidance by IDSA suggested high-dose ampicillin-sulbactam as a component of combination therapy for CRAB, regardless of whether susceptibility has been demonstrated (Tamma et al., 2023). In our study, seven patients received therapy with sulbactam. When CRAB infections were refractory to other antibiotics or in cases where intolerance or resistance to other agents, cefiderocol may also be an option (Pintado et al., 2023; Tamma et al., 2023).

In our study, 10 patients (58.82%) were ST, and seven (41.18%) were FT. Few studies have investigated the differences between FT and ST. Chen et al. reported that for nosocomial meningitis and patients with multidrug-resistant gram-negative bacteria, significantly different factors in ST vs FT included pathogen type, highest body temperature in the first 24 h of symptoms, CSF glucose level and meropenem susceptibility (for AB) (Chen et al., 2020). In our study, univariate analyses revealed that mechanical ventilation, routine white blood cell counts in the peripheral blood, PCT, protein in the CSF, the proportion of septic shock and CRAB differed significantly between the ST and FT groups. Standard laboratory parameters, such as peripheral leukocyte counts, glucose levels and protein in the CSF were not reliable predictors

for infection in patients with healthcare-associated meningitis (Tunkel et al., 2017). However, in this study, patients with FT had lower peripheral leukocyte counts and higher CSF protein levels than did those with ST ($P=0.031$, 0.032). These laboratory parameters can be used as indicators to evaluate the disease severity, and these indicators should draw clinicians' attention to these patients.

All patients in the FT group had CRAB. Due to the limited antimicrobial drugs available, CRAB tends to cause severer infections and has a worse prognosis than CSAB (Pogue et al., 2022). However, we asked whether the clinical manifestations differed between these two groups and compared their clinical characteristics. CRAB infections mostly occurred in the ICU, whereas CSAB infections mostly occurred in the general ward, consistent with the results of previous studies (Zhang et al., 2021). The widespread prevalence of CRAB in ICUs is usually related to the large number of severe patients with low immunity, catheter and mechanical ventilation and wide antibiotic use (Kyriakidis et al., 2021). The proportion of CSF sterilization was significantly lower in the CRAB group than in the CSAB group, which was understandable given the limited selection of effective antibiotics for CRAB. Notably, protein levels in the CSF were significantly higher in the CRAB group than in the CSAB group. We speculated whether this was related to the virulence differences between CRAB and CSAB but have found no other studies reporting similar results; thus, the clinical significance of our results requires further exploration.

This study had some limitations. First, the major limitation was the single-center, retrospective nature of the study. Collecting complete data was difficult and may have led to biased results. Moreover, no independent risk factor analysis was performed owing to the limited number of cases. Second, because the CRAB epidemiology differs regionally and may also vary over time in the same region, conclusions from our study may not be universally applicable to other centers.

In conclusion, AB meningitis in children was mostly secondary to neurosurgery and had a high mortality rate. FT was associated with mechanical ventilation, septic shock, CRAB, lower peripheral leukocyte counts, higher protein in the CSF and PCT. Larger studies are needed to identify independent risk factors for adverse outcomes. Because of the emergence of CRAB, choosing the best antibiotics remains challenging and critical to patient outcomes.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Children's Hospital of Fudan University. The studies were conducted in accordance with the local legislation and

institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

ZW: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. LY: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. PF: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. XW: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. JX: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. YY: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. SH: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. CW: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. HY: Methodology, Project administration, Resources, Writing – review & editing.

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Conflict of interest

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

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Recombinase-aided amplification assay for rapid detection of imipenem-resistant *Pseudomonas aeruginosa* and rifampin-resistant *Pseudomonas aeruginosa*

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The indiscriminate use of antibiotics has resulted in a growing resistance to drugs in *Pseudomonas aeruginosa*. The identification of antibiotic resistance genes holds considerable clinical significance for prompt diagnosis. In this study, we established and optimized a Recombinase-Aided Amplification (RAA) assay to detect two genes associated with drug resistance, *oprD* and *arr*, in 101 clinically collected *P. aeruginosa* isolates. Through screening for the detection or absence of *oprD* and *arr*, the results showed that there were 52 Imipenem-resistant *P. aeruginosa* (IRPA) strains and 23 Rifampin-resistant *P. aeruginosa* (RRPA) strains. This method demonstrated excellent detection performance even when the sample concentration is 10 copies/μL at isothermal conditions and the results could be obtained within 20 minutes. The detection results were in accordance with the results of conventional PCR and Real-time PCR. The detection outcomes of the *arr* gene were consistently with the resistance spectrum. However, the antimicrobial susceptibility results revealed that 65 strains were resistant to imipenem, while 49 strains sensitive to imipenem with *oprD* were identified. This discrepancy could be attributed to genetic mutations. In summary, the RAA has higher sensitivity, shorter time, and lower-cost instrument requirements than traditional detection methods. In addition, to analyze the epidemiological characteristics of the aforementioned drug-resistant strains, we conducted Multilocus Sequence Typing (MLST), virulence gene, and antimicrobial susceptibility testing. MLST analysis showed a strong correlation between the sequence types ST-1639, ST-639, ST-184 and IRPA, while ST-261 was the main subtype of RRPA. It was observed that these drug-resistant strains all possess five or more virulence genes, among which *exoS* and *exoU* do not coexist, and they are all multidrug-resistant strains. The non-coexistence of *exoU* and *exoS* in *P. aeruginosa* is related to various factors including bacterial regulatory mechanisms and pathogenic mechanisms. This indicates that the relationship between the presence of virulence genes and the severity of patient infection is worthy of attention. In conclusion, we have

developed a rapid and efficient RAA (Recombinase-Aided Amplification) detection method that offers significant advantages in terms of speed, simplicity, and cost-effectiveness (especially in time and equipment aspect). This novel approach is designed to meet the demands of clinical diagnostics.

KEYWORDS

Pseudomonas aeruginosa, recombinase-aided amplification, rapid detection, antimicrobial susceptibility testing, ARR, OprD

1 Introduction

Pseudomonas aeruginosa, a prevalent opportunistic pathogen within the family Pseudomonadaceae, stands as a significant causative agent of hospital-acquired infections (HAIs) in tertiary hospitals (Logan et al., 2017; Curran et al., 2018). Infections caused by *P. aeruginosa* can manifest in various areas, including the lungs, urinary tract, wounds, blood (such as septicemia), eyes, and other diseases like pneumonia (Lee et al., 2018; Tacconelli et al., 2018). *P. aeruginosa* is widely distributed, it is typically non-pathogenic under normal circumstances. However, the infection rate of *P. aeruginosa* increases when the host's immune system is weakened (Prithiviraj et al., 2005; Bassetti et al., 2017). In the intensive care unit (ICU), *P. aeruginosa* infections are linked to high incidence and mortality rates across various populations, including individuals with chronic obstructive pulmonary disease and cystic fibrosis (Diekema et al., 2000; Cui et al., 2022). The entire genome size of *P. aeruginosa* ranges from 5.5 to 7 Mbp, showcasing robust genetic coding capabilities that contribute to metabolic diversity and drug resistance (Montgomery et al., 2018). The spread of multidrug-resistant strains has made treating diseases caused by those pathogens increasingly challenging (Burrows, 2018; Arbune et al., 2021). Mechanisms of antibiotic resistance in *P. aeruginosa* include natural resistance, acquired resistance, and adaptive resistance (Shen and Fang, 2015; Chevalier et al., 2017). IRPA and RRA exhibit resistance to various antibiotics and robust survivability in harsh environments such as hospitals, posing significant challenges to clinical treatment (Abniki et al., 2024). The overuse of antibiotics has contributed to the emergence of strains resistant to imipenem and rifampicin. According to Hamid Vaez et al., IRPA prevalence in Iran was reported at 54% (Vaez et al., 2017). Suwantararat et al. reported IRPA prevalence in Southeast Asian countries, noting it was 31.1% in Philippines (Suwantararat and Carroll, 2016). Rifampicin, a derivative of rifamycin, exhibits a broad spectrum of antibacterial activity against Gram-positive bacteria. The lower prevalence of IRPA isolates could be attributed to the limited use of rifampicin antibiotics in these regions (Liu et al., 2020).

Currently, in addition to traditional methods, laboratories and clinical platforms have also established immunological methods based on antigen-antibody specific binding and molecular biological methods (DNA sequencing technology, PCR-based technology, new molecular detection technology) for pathogen detection (Rajapaksha et al., 2019). However, many of these methods are time-consuming and cannot be easily implemented in primary-level laboratories (Bonetta et al., 2016; Yoon et al., 2021).

RAA is based on the recombinase polymerase amplification (RPA) technology. RPA was initially introduced by Niall Armes in 2006 (Cambridge, United Kingdom, founded by the Wellcome Trust Sanger Institute) (Piepenburg et al., 2006; Li et al., 2018). RAA is developed by TwistDx in the UK and further advanced by Jiangsu Qitian Gene Biotechnology (Fan et al., 2020; Li et al., 2021), has become a molecular tool widely used in the identification of genes of various pathogens (Mao et al., 2022; Yan et al., 2023).

RAA employs single-strand binding proteins, DNA polymerase, and recombinases to amplify nucleic acids at an isothermal temperature (38–41°C) (Wu et al., 2021). This technology uses recombinases from *Escherichia coli*, allowing for tight binding with primer DNA at room temperature, forming an enzyme-primer aggregate (Song et al., 2018). The single-stranded DNA-binding protein aids in unraveling the double-stranded structure of the template DNA, and the DNA polymerase catalyzes the formation of new complementary DNA strands, resulting in exponential growth of the amplification product (Zhao et al., 2020). With the addition of a fluorescence probe, RAA can perform Real-time detection, and the results can be obtained in just 5–20 minutes with high sensitivity (Shelite et al., 2021; Cao et al., 2023).

In this paper, we have established a high-speed platform based on RAA technology to detect the resistance genes *oprD/arr* in *P. aeruginosa*. This platform enables efficient and rapid detection of resistance genes, with results attainable in just 10 minutes and a detection sensitivity of 10 copies/μL. The epidemiological analysis revealed that both IRPA and RRA contain more than five virulence genes, and all strains tested exhibited multidrug resistance. IRPA of ST-1639 and RRA of ST-261 are the most commonly identified types.

2 Materials and methods

2.1 Bacterial strains and clinical isolates

A total of 101 clinical antibiotics-resistance samples of *P. aeruginosa* were collected from the Xi'an Children's Hospital of Shaanxi Province. These samples included 92 sputum samples, 3 urine samples, 3 blood samples, 2 bronchoalveolar lavage fluid samples, and 1 skin secretion sample. Isolates were obtained from the above clinical specimens. The strains tested were indeed cultured as isolates before testing with the RAA method. All isolated strains underwent bacterial identification. Isolates were obtained from various clinical specimen types listed. PAO1 (stored in our laboratory) was used as the wild type strain in this experiment.

2.2 DNA extraction

The total DNA of the bacterial strains was extracted using the Sangon Quick Bacterial Genomic DNA Isolation Kit (Sangon Biotech, China). The DNA was eluted with 100 μ L of enzyme-free water and stored at -80°C for future use.

2.3 Preparation of recombinant plasmids

Different data sources of National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) and Pseudomonas Genome DB (<https://www.pseudomonas.com>) were used to further screen the drug-resistant genes *oprD* and *arr* as target genes. The full sequences of *oprD* (1332 bp) and *arr* (1578 bp) corresponding to the nucleotide sequences of *P. aeruginosa* (PAO1, NC_002516) were cloned into the Pme6032 vector (Our laboratory stored). The number of copies of the recombinant plasmid was calculated using plasmid concentration measured with Nanodrop One (Thermo Fisher Scientific, Waltham, MA, United States). The recombinant plasmid is diluted from 10^7 copies/ μ L to 10^0 copies/ μ L using the following formula, DNA copies (copies/ μ L) = $[6.02 \times 10^{23} \times \text{plasmid concentration (ng}/\mu\text{L}) \times 10^{-9}] / [\text{DNA length (bp)} \times 660]$, stored at -80°C for following detection.

$$\text{DNA copies (copies}/\mu\text{L}) = \frac{6.02 \times 10^{23} \times \text{plasmid concentration (ng}/\mu\text{L}) \times 10^{-9}}{[\text{DNA length (bp)} \times 660]}$$

2.4 RAA primer and probe design

The screening of *oprD* and *arr* gene sequences was conducted using data retrieved from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). The principles of RAA primer and probe design are as follows: the primers range from 30 to 35 base pairs (bp) in length, while the probes range from 46 to 52 bp. The size of the RAA amplification product falls within the range of 100 to 200 bp. Both the probes and primers are designed to target

conserved regions of the gene. The specificity of primers and probes was confirmed using NCBI's Primer-BLAST program. RAA-*oprD*-primer1-fw (30 bp), RAA-*oprD*-primer1-rv (32 bp), RAA-*oprD*-probe2 (41 bp) were selected as the primers and probes for IRPA detection; RAA-*arr*-primer3-fw (30 bp), RAA-*arr*-primer3-rv (32 bp) and RAA-*arr*-probe2 (44 bp) serves as primer and probe for RRA detection. Additionally, conserved regions of the 16S rRNA gene were selected for the design of internal positive controls. All primers and probes are synthesized and purified using Biotech (Shanghai, China) by high performance liquid chromatography.

2.5 PCR and real-time PCR

The 20 μ L reaction volume contains the following components for all PCR: 10 μ L PCR master mix reagents (2 $\times$ SanTaq Fast PCR Master Mix, Beijing, China), 8 μ L of Sterile water, 0.5 μ L of 10 μ M *oprD*-fw (or *arr*-fw) primers and *oprD*-rv (or *arr*-rv) primers, and 1 μ L DNA templates. The primer sequences are showed in Table 1.

Real-time PCR based on the *oprD* gene for the detection of oligonucleotide sequences of *oprD* forward primers (30-50 bp), *oprD* reverse primers (30-50 bp), and *oprD*-probe (FAM, BHQ1). Real-time PCR based on the *arr* gene for the detection of oligonucleotide sequences of *arr* forward primers (30-50 bp), *arr* reverse primers (30-50 bp), and *arr*-probe (FAM, BHQ1). The reactions were prepared as a 25 μ L reaction volume containing 12.5 μ L TaqMan Universal Master Mix, 0.5 μ L forward primers, 0.5 μ L reverse primers, 8.5 μ L double distilled water, 1 μ L probe and 2 μ L extracted DNA. The concentration of primer and probe was 10 μ M. A positive sample is defined as a sample with a period threshold (CT) value < 30. The primer and probe sequences are listed in Table 1.

2.6 RAA technique

We used the RAA kit (Qitian, China) to conduct the RAA assay. The reaction system consisted of 50 μ L, including 2.1 μ L of forward primer, 2.1 μ L of reverse primer, and 0.6 μ L of probe, all at a concentration of 10 μ M. Additionally, reaction buffer (25 μ L), magnesium acetate (2.5 μ L of 280 mM), deionized water (15.7 μ L), and 2 μ L of template DNA were included. Note that due to the high sensitivity of RAA detection and to avoid contamination, template DNA should be added last. The reaction mixture was briefly centrifuged and mixed in RAA-B6100—an isothermal shaking incubator (QiTian, Wuxi, China) at 39°C for 4 minutes. Subsequently, the mixture was placed in RAA-F1620—a fluorescence detector (QiTian, Wuxi, China) to measure FAM fluorescence signals every 20 seconds.

2.7 Phenotype analysis method of IRPA and RRA

Typically, this experiment involves cultivating bacteria in media containing varying concentrations of antibiotics, followed by the

TABLE 1 Primers used in this research.

Primer/ probe	Sequence (5'- 3')
<i>oprD</i> -p-fw	CCCAGAACCTTTTATATTTGT
<i>oprD</i> -p-rv	CACGGTAACGCATTAAACGA
pcr- <i>oprD</i> -fw	CCCAGAACCTTTTATATTTGT
pcr- <i>oprD</i> -rv	CACGGTAACGCATTAAACGA
Rtpcr- <i>oprD</i> -fw	AATCCTGGCAGGCTCGCTACG
Rtpcr- <i>oprD</i> -rv	GCCAAGGACCTGTCGTTCCGC
Rtpcr- <i>oprD</i> -probe	FAM-CCAAGATGTCTGACAACAAC-BHQ1
RAA- <i>oprD</i> -primer1-fw	CCTGACTTTCATGGTCCGCTATATCAATGG
RAA- <i>oprD</i> -primer1-rv	CGTTCGTGGTGCTTTGGTTGGAGCTTCGGTTC
RAA- <i>oprD</i> -probe2	TGGCACCAAGATGTCTGACAACAACGTCGGC[FAM-dT] [THF][BHQ-dT]AAGAACTACG [3'-block]
<i>arr</i> -primer-fw	GCAATTAGAGGGAAGATGAG
<i>arr</i> -primer-rv	TTCAGGCTATTGGACGAGTT
pcr- <i>arr</i> -fw	GCAATTAGAGGGAAGATGAG
pcr- <i>arr</i> -rv	TTCAGGCTATTGGACGAGTT
Rtpcr- <i>arr</i> -fw	CCATCTCTCATGATAATTACA
Rtpcr- <i>arr</i> -rv	AGTGACATAGCAAGTTCAGC
Rtpcr- <i>arr</i> -probe	FAM-TTGGTGACTTGCTAACCACA-BHQ1
RAA- <i>arr</i> -primer3-fw	TTCCCATCTCTCATGATAATTACAAGCAGG
RAA- <i>arr</i> -primer3-rv	CGACTTGAACGATACAGTGACAGACCGGAGCT
RAA- <i>arr</i> -probe2	CATGTTAACATAGATGTCATAATCACACCC[FAM-dt] [THF][BHQ-dt]AGGATAAAACCGCC [3'-block]

FAM, 6-carboxyfluorescein; THF, Tetrahydrofuran; BHQ, Black hole quencher; 3'-block, 3'-phosphate blocker; fw, forward primer; rv, reverse primer; P, probe; Rtpcr, Real-time PCR.

observation of bacterial growth under different antibiotic conditions (Blair et al., 2015; Denis et al., 2019). The antimicrobial susceptibility testing for the chosen strains were conducted through the disk diffusion method, and the results were interpreted following the guidelines provided by the Clinical and Laboratory Standards Institute. The antibiotics used include ciprofloxacin (CIP), tobramycin (TOB), cefepime (SCF), aztreonam (AZT), polymyxin (PMB), piperacillin (PIP), meropenem (MEM), imipenem (IPM), cefepime (FEP),

ceftazidime (CAZ), levofloxacin (LEV), gentamicin (GN), Rifampin (RFP), and amikacin (AMK).

2.8 Determination of MLST and virulence factor

MLST analysis of *P. aeruginosa* is conducted by analyzing the sequence variations of housekeeping genes (*nuoD*, *mutL*, *trpE*, *acsA*, *aroE*, *guaA* and *ppsA*). The PCR products are sent to Shanghai Sangon Biotech for sequencing, and the sequencing results are submitted to the *P. aeruginosa* MLST database (<https://pubmlst.org/paeruginosa/>) for analysis. Strain types that do not match existing databases will be identified as new sequence typing (ST). Virulence analysis using the genes encompassed *plcH*, *aprA*, *algD*, *exoS*, *exoT*, *exoU*, *exoY*, *toxA* and *nor*.

2.9 Technical route of this study

This experiment primarily utilizes RAA as the detection method, with the technical route shown in 178 Figure 1. From gene screening to RAA detection, we have gone through the following steps: (1) Following the isolation and identification of clinical bacterial strains, collect and culture bacteria and store them in a strain storage center. (2) Antimicrobial susceptibility testing and PCR for drug-resistant isolates. (3) Selection of genes: drug-resistant genes consistent with the resistant phenotype will be selected for RAA detection and optimization. (4) High temperature denaturation extraction of bacterial DNA. (5) RAA detection. RAA is a novel isothermal nucleic acid amplification technology that requires the design of specific primers and probes. Visual analysis can be achieved by a fluorescence detector (QT-RAA-1620; Jiangsu Qitian Bio-Tech Co., Ltd., China) (6).

2.10 Statistical analysis

Statistical analysis was performed using SPSS 21.0 (IBM, Armonk, NY, USA). Probit analysis for the detection limit of the PCR, Real-time PCR and RAA was performed at a 95% probability level. We performed repeated experiments and calculated p-values and kappa values for RAA, PCR, and Real time PCR.

3 Results

RAA technology performs DNA amplification at isothermal temperature, significantly saving time and cost. Here, we established RAA technology to detect *oprD* (outer membrane channel protein

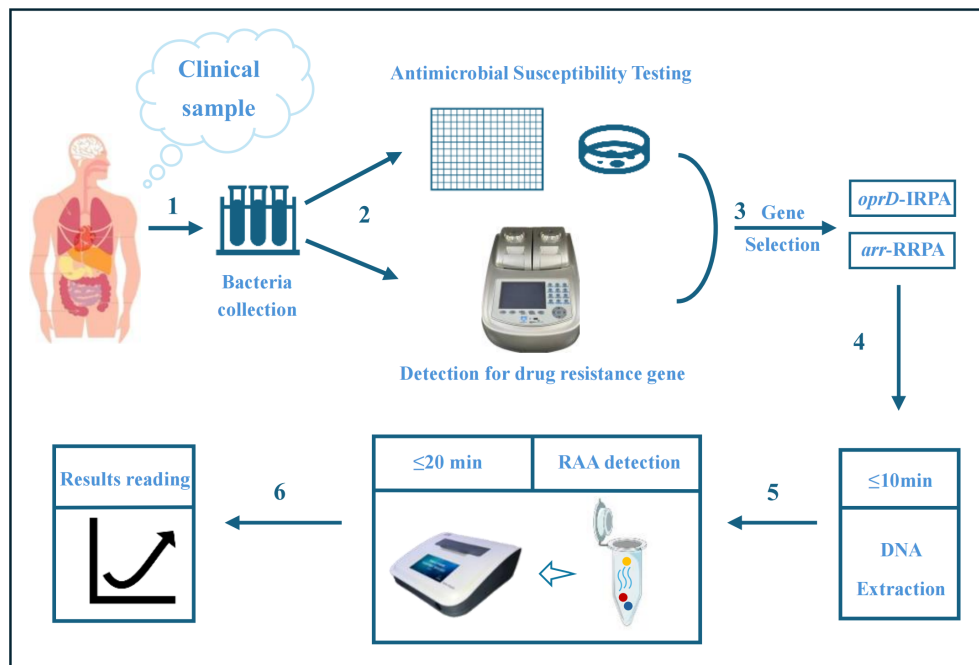


FIGURE 1

Technical route of this study. (I) Clinical isolates are collected and cultured. (II) Antimicrobial susceptibility testing and PCR for drug-resistant genes. (III) Selection of genes. (IV) High temperature denaturation extraction of bacterial DNA. (V-VI) Detection of *oprD/arr* by RAA. The blue curve represents the template DNA, the blue circle represents the probe, the red circle represents the primer, and the orange represents the recombinant enzyme medium. The reaction results can be detected with a constant temperature instrument.

genes) or *arr* (aminoglycoside response regulator gene) rapidly and used it to analyze the clinical samples of antibiotic-resistant *P. aeruginosa*.

3.1 Optimizing the RAA assay-primer, probe and temperature

RAA detection exhibits high sensitivity towards target sequences, enabling reliable detection in complex samples. We designed primers and probes for RAA, utilizing recombinant

oprD or *arr* plasmids as positive templates and sterile water as negative templates to optimize the detection of RAA. Five sets of primers (RAA-*oprD*-primer1, RAA-*oprD*-primer2, RAA-*oprD*-primer3, RAA-*oprD*-primer4, RAA-*oprD*-primer5) and two probes (RAA-*oprD*-probe1, RAA-*oprD*-probe2) were designed in the conserved region of the gene, and the amplification efficiency was determined based on the change in fluorescence intensity at 492 nm. Finally, we selected RAA-*oprD*-primer1 as the best primer and RAA-*oprD*-probe2 as the best probe. The same method was used for the optimization of *arr*, and RAA-*arr*-primer3 and RAA-*arr*-probe2 were finally selected (Table 1; Figure 2).



FIGURE 2

Selection of specific regions and primer positions for RAA assay. (A) Primer and probe set to amplify the *oprD* gene. (B) Primer and probe set to amplify the *arr* gene. Probes are indicated by blue, primer are indicated by orange.

The suitable reaction temperature range for the Recombinase is 38–42°C (Yan et al., 2023). Next, we used primer pairs of RAA-*oprD*-primer1 or RAA-*arr*-primer3, adjusted the reaction temperature to compare the amplification efficiency of RAA assay, and chose appropriate reaction temperature within 50 μ L system. The results showed that the fluorescence generated by RAA at 39°C was significantly higher than that at other temperatures. Therefore, 39°C was the optimal temperature for our system with the highest amplification efficiency (Figures 3A, B). In addition, the amplification efficiency under different probe concentrations was compared, and the probe concentration selected in our system was 10 μ M (Figures 3C, D).

3.2 Sensitivity for RAA detection

Next, we used RAA technology to detect the *oprD* or *arr* to determine the sensitivity of RAA detection. In the experiment, we diluted the constructed recombinant plasmids containing *oprD* or *arr* from 10^7 copies to 10^0 copies respectively to detect the fluorescence signal (Figure 4). We found that clear signals could be detected using 10 copies/reaction (Figures 4A, B), whereas PCR detection required 10^3 copies/reaction (Figures 4C, D), which is similar to previous reports (Fu et al., 2022).

3.3 Analytical specificity for RAA detection

Subsequently, we tested the specificity of *oprD* or *arr* using the RAA platform, and as a control, we also detected 7 other resistance genes including *msr*, *tetA*, *mph*, etc. As shown in Figure 5, only strains containing *oprD* or *arr* were able to exhibit fluorescence

signals. Also, we amplified the standard strain with PCR primers and detected it by agarose gel electrophoresis. We found that only strains with *oprD* or *arr* showed specific amplification bands, which was consistent with the results of the RAA test. This confirmed the specificity of RAA detection.

3.4 Clinical sample detection by RAA assay

After establishing the RAA detection technology, we simultaneously used conventional PCR, Real-time PCR and RAA technique to detect *P. aeruginosa* in 101 samples collected from Xi'an Children's Hospital (Shaanxi, China). Compared with PCR and Real-time PCR methods, RAA technology has comparable detection accuracy for *oprD* or *arr* genes, but the time is significantly shortened (Tables 2A, 2B) (Figure 6). PCR and Real-time methods typically take 2 hours, while the RAA method takes less than 20 minutes.

Among the 101 clinical antibiotic-resistant *P. aeruginosa* isolates collected, we detected 23 strains that contained the *arr* gene and showed resistant to Rifampin, which was highly consistent with the results obtained by other methods. However, the results for detection of *oprD* did not match the antimicrobial susceptibility results. There should be 52 strains of *P. aeruginosa* that are resistant to imipenem based on the lack of detection of the *oprD* gene, but antimicrobial susceptibility testing results indicated 65 IRPA strains. The previous reports indicate this discrepancy may be associated with mutations or deletions in the *oprD* gene and other factors. The absence or mutation of *oprD* leads to the closure of bacterial channels, impeding the entry of imipenem into bacteria and inhibiting its effects, consequently resulting in the development of resistance to imipenem (Suresh et al., 2020; Gonzalez-Vazquez et al., 2021).

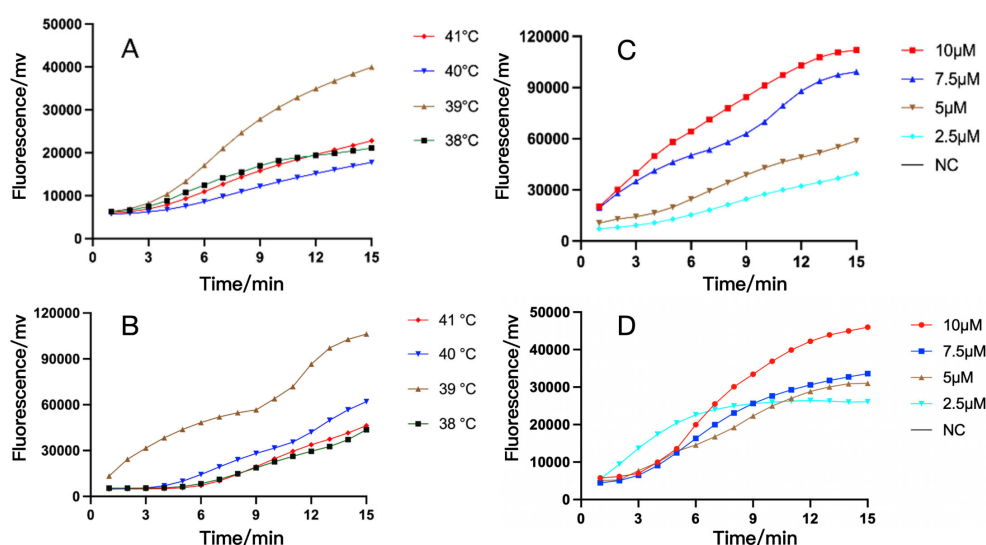


FIGURE 3

The optimization of temperature and probe concentration of RAA assay for IRPA and RRA detection. (A, B) The RAA analysis of *oprD* gene at different temperature (A) and different probe concentration (B). (C, D) The RAA analysis of *arr* gene at different temperature (C) and different probe concentration (D). The results showed that their optimal temperature is 39°C and the optimal probe amplification concentration is 10 μ M. NC, Negative Control.

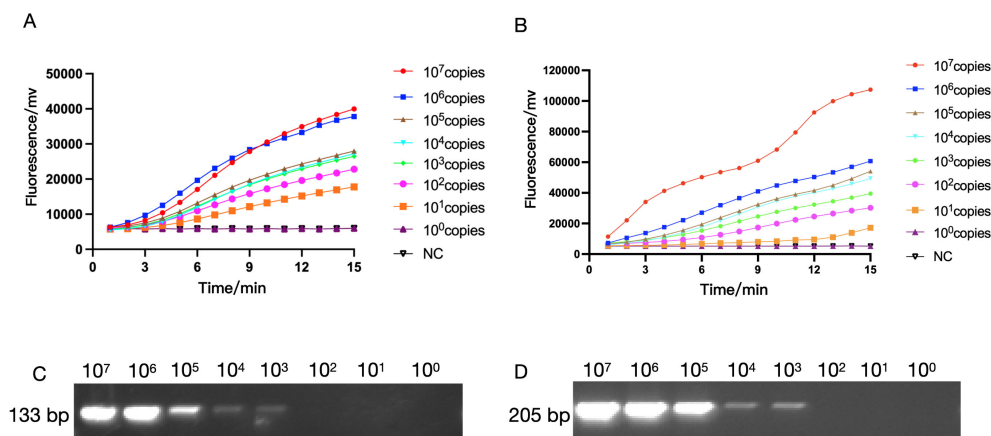


FIGURE 4

The sensitivity of RAA and PCR assay for IRPA and RRPA detection. The sensitivity of RAA analysis using the primer and probe set RAA-*oprD*-primer1 (A), RAA-*arr*-primer2 (B). The plasmid ranged from 1×10^0 copies/ μ L to 1×10^7 copies/ μ L. NC, Negative Control. The sensitivity of PCR for IRPA and RRPA detection. (C, D) The sensitivity of RAA and conventional PCR detection.

Mutations in the *oprD* gene are the primary cause of *P. aeruginosa*'s resistance to imipenem. We also analyzed the types of *oprD* mutations in this batch of strains. The mutation sites were mainly located the transmembrane region, loop 2, and loop 3 of the protein (Table 3), which is similar to the results of previous reports (Ochs et al., 2000; Li et al., 2012).

3.5 Antimicrobial susceptibility analysis

Furthermore, we carried out the antimicrobial susceptibility testing for IRPA and RRPA. Among the 65 IRPA strains, 85.5% (55 strains) were resistant to meropenem, 67.8% (44 strains) were resistant to levofloxacin, 58.5% (38 strains) were resistant to aztreonam, and 50.8% (33 strains) were resistant to ciprofloxacin, ceftazidime, cefepime and piperacillin/tazobactam combination (Table 4).

The 101 clinical strains of *P. aeruginosa* collected include 23 strains of RRPA. The antimicrobial susceptibility tests indicate that among them, 17 strains (73.9%) were resistant to meropenem, 16 strains (69.6%) were resistant to piperacillin/tazobactam combination, and 14 strains (60.9%) were resistant to imipenem (Table 5).

The results showed that most of the clinical antibiotic-resistant *P. aeruginosa* collected were multidrug-resistant. The most of imipenem, meropenem, and piperacillin-resistant *P. aeruginosa* also exhibit resistance to levofloxacin.

3.6 Molecular characteristics of the IRPA and RRPA isolates

To understand the distribution and epidemiological characteristics of the pathogen, Multilocus Sequence Typing (MLST) and antimicrobial susceptibility testing was used to classify IRPA and RRPA. MLST analysis was conducted on 65 strains of IRPA and the results revealed that ST-1639 is the predominant genotype, followed by ST-261, ST-485, ST-2375, and ST-2389. Among the 23 isolated strains of RRPA, a total of 12 ST types were identified. Notably, ST-261 was the predominant genotype, accounting for 30.43% of the strains. Particularly interesting is the discovery of one RRPA strain that exhibited resistance to all tested antibiotics, and its ST type was identified as ST-639. The statistical results are shown in the Figure 7, Supplementary Tables 1 and 2.

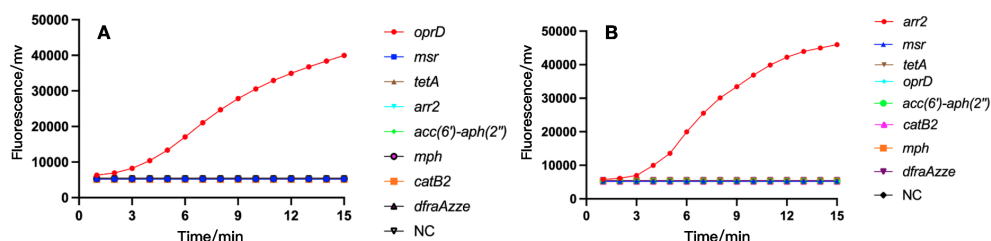


FIGURE 5

The specificity of RAA assay for IRPA (A) and RRPA (B) detection. Only the recombinant plasmids produced amplification signals, whereas the negative control and control bacterial samples produced negative amplification signals. NC, Negative Control.

TABLE 2A Comparison of recombinant enzyme-assisted amplification methods (RAA) with PCR and real-time PCR for the detection of Imipenem-resistant *P. aeruginosa* (IRPA).

	RAA	PCR	Real-time PCR	Antimicrobial Susceptibility Testing
IRPA (<i>oprD</i> neg)	52 (5–20 min)	52 (2 h)	52 (2 h)	65 (2 d)
ISPA (<i>oprD</i> pos)	49 (5–20 min)	49 (2 h)	49 (2 h)	49 (2 d)

Through screening of drug-resistant phenotypes and genes in the early stage, it was found that the *oprD*-positive strains (49 strains) were imipenem-sensitive *Pseudomonas aeruginosa* (49 strains), so there are 52 IRPAs.

TABLE 2B Comparison of recombinant enzyme-assisted amplification methods (RAA) with PCR and real-time PCR for the detection of rifampicin resistant *P. aeruginosa* (RRPA).

	RAA	PCR	Real-time PCR	Antimicrobial Susceptibility Testing
RRPA (<i>arr</i> pos)	23 (5–20 min)	23 (2 h)	23 (2 h)	23 (2 d)
RSPA (<i>arr</i> neg)	23 (5–20 min)	23 (2 h)	23 (2 h)	23 (2 d)

Through screening of drug-resistant phenotypes and genes in the early stage, it was found that the *arr*-positive strains (23 strains) were RRPA (23 strains).

3.7 Virulence factors of the *P. aeruginosa* isolates

We utilized PCR to ascertain the presence of virulence factors (*exoY*, *exoS*, *exoT*, *exoU*, *plcH*, *aprA*, *pys*, *toxA*) in clinical isolates. The *aprA* gene is associated with T1SS, while *toxA* and *plcH* are associated with T2SS, and *exoS*, *exoT*, *exoY*, and *exoU* are associated with T3SS. Positive IRPA virulence gene detections encompass 11 types, with all multidrug-resistant *P. aeruginosa* isolates carrying at least 5 virulence genes. The detection rate of the *toxA* gene associated with Type II Secretion System is 100%. Its virulence is modulated by three secretion systems, particularly exerting significant influence on IRPA virulence, especially those associated with the T3SS secretion system (*exoY*, *exoS*, *exoT*) (Supplementary Table 1). All

virulence factors in RRA showed relatively high detection rates among the 23 strains, with all containing *toxA*. The *toxA* gene is secreted via T2SS, resulting in modification and loss of intracellular proteins. In order of detection rates, virulence genes are arranged as follows: *toxA*, *algD*, *aprA*, *exoY*, *norC* (Supplementary Table 1).

4 Discussion

P. aeruginosa causes various diseases and localized infections resulting from surgical procedures or burns often lead to fatal injury (Dunn and Wunderink, 1995). Multidrug-resistant *P. aeruginosa* (MDR-PA) is commonly identified in major hospitals worldwide and the Centers for Disease Control and Prevention (CDC) has categorized MDR-PA as a significant menace (Kang et al., 2003; Karruli et al., 2023). Antibiotic resistance, especially multidrug resistance, is one of the most critical factors in patients acquiring infections with MDR-PA (Denis et al., 2019; Naik et al., 2021). The rapid diagnosis of antibiotic resistance genes aims to detect whether patients carry or possess specific drug-resistant gene characteristics related to certain diseases, and timely diagnosis can avoid unnecessary antibiotic use and treatment delays, thereby reducing healthcare cost and conserving medical resources.

RAA demonstrates excellent detection capability and this method not only saves time but also does not require complex instrumentation. Due to the extremely high sensitivity of the RAA detection method we developed, it can directly test the sputum or alveolar lavage fluid of infected patients without the need for complex and time-consuming cultivation. This process can be completed within 20 minutes, ensuring rapid turnaround times. Furthermore, the visualization of results significantly enhances the efficiency of doctors' diagnoses and minimizes patient waiting times to the greatest extent possible. As an initial attempt, we selected the *oprD* and *arr* genes of *P. aeruginosa* to test the characteristics of RAA in rapidly identifying resistance genes. OprD protein plays a role as a specific channel protein for the rapid entry of imipenem into *P. aeruginosa* and decreased expression or loss of the *oprD* lead to imipenem resistance (Quinn et al., 1991; Sun et al., 2016), and the aminoglycoside response regulator gene *arr* can deactivate rifampicin through ribosylation, which is a crucial factor in

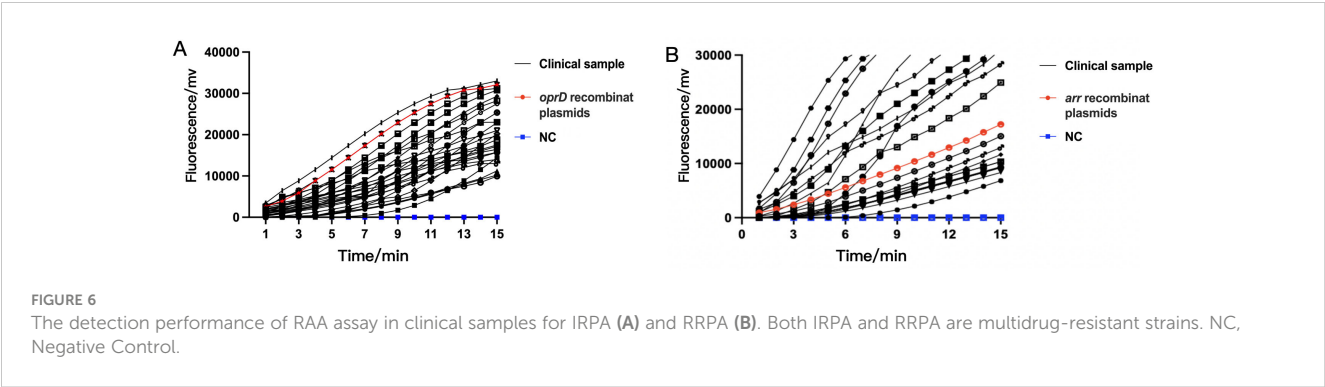


TABLE 3 IRPA-*oprD* gene mutation type.

	Mutation Sites	Amino Acid Sites	Mutation Region
Isolate 1	c.344A>C	p.K115T	Loop 2
Isolate 2	c.508T>C	p.F170L	Loop 3
Isolate 3	c.471G>C	p.A157P	Loop 3
Isolate 4	c.826A>G	p.T276A	Transmembrane region
Isolate 5	c.308C>G	p.T103S	Loop 2
Isolate 6	c.379G>C	p.V115T	Loop 2
Isolate 7	c.944_945CA>GC	p.V127L	Loop 6
Isolate 8	c.565_566TG>AC	p.V189T	Loop 3
Isolate 9	c.719G>C	p.S240T	Transmembrane region
Isolate 10	c.553G>C	p.E185Q	Transmembrane region
Isolate 11	c.556_558CCG>GGC	p.P186G	Loop 3
Isolate 12	c.308C>G	p.T103S	Loop 2
Isolate 13	c.886A>C	p.K296Q	Transmembrane region

bacterial resistance to rifampicin (Tribuddharat and Fennewald, 1999; Arlet et al., 2001; Naas et al., 2001; Alexander et al., 2003).

In clinical practice, the detection of drug-resistant genes traditionally relies on comprehensive antimicrobial susceptibility testing, which is time-consuming and costly (Shen and Fang, 2015; Holbrook and Garneau-Tsodikova, 2018). We conducted a comparative analysis of RAA, conventional PCR, and Real-time PCR, examining factors such as cost, sensitivity, scope of application, and their respective strengths and weaknesses (Table 6). The results showed that RAA has obvious advantages. When we apply RAA assay to clinical gene diagnosis and commercialize it, it can replace the time-consuming and labor-intensive traditional detection methods, which can greatly improve the accuracy of gene diagnosis, and help clinicians make quick decisions. In this study, we use RAA method to detected *oprD* and *arr* genes. The results indicate that the minimum detection limit of the RAA assay is 10 copies/reaction, which approaches the highest sensitivity of most Real-time PCR methods. Furthermore, the RAA demonstrated specificity in detecting IRPA and RRP A without cross-reactivity with other antibiotic resistance genes in *P. aeruginosa*, such as *ges* and *msr*. In order to assess the clinical feasibility of this method, 101 clinical isolates of *P. aeruginosa* were tested using the RAA assay, conventional PCR, and TaqMan probe-based Real-time PCR. The result of gene detection for *oprD* and *arr* are consistent with those obtained from conventional PCR and Real-time PCR. This indicates that the RAA method exhibits good sensitivity comparable to PCR and Real-time PCR, but in a much shorter time. RAA can be completed in 20 minutes, while PCR and Real-time PCR require about 2 hours. Therefore, the RAA assay established in our study has the potential to be developed into a

TABLE 4 Phenotypic analysis of 65 IRPA isolates.

Types of antibiotics	Antibiotic	Number	Percentage
Aminoglycoside	Amikacin	2	3.1%
	Gentamicin	11	16.9%
	Tobramycin	5	7.7%
Quinolones	Ciprofloxacin	33	50.8%
	Levofloxacin	44	67.8%
Polypeptides	Ploymyxin B	0	0%
Monolactams	Aztreonam	38	58.5%
Cephalosporins	Ceftazidime	33	50.8%
Fourth-generation cephalosporin	Cefepime	33	50.8%
Carbapenems	Meropenem	55	85.5%
β -lactam	Piperacillin/tazobactam	33	50.8%
	Cefepime/sulbactam	31	47.7%
Macrolide antibiotics	Rifampin	9	13.8%

portable on-site kit for rapid screening of IRPA and RRP A in basic clinical laboratories.

The genetic testing of RRP A is consistent with the results of drug susceptibility testing, indicating a higher correlation between *arr* and drug-resistant phenotypes. As has been previously reported,

TABLE 5 Phenotypic analysis of 23 RRP A isolates.

Types of antibiotics	Antibiotic	Number	Percentage
Aminoglycoside	Amikacin	1	4.3%
	Gentamicin	7	30.4%
	Tobramycin	2	8.8%
Quinolones	Ciprofloxacin	8	34.8%
	Levofloxacin	12	52.2%
Polypeptides	Ploymyxin B	0	0%
Monolactams	Aztreonam	9	58.5%
Cephalosporins	Ceftazidime	11	39.1%
Fourth-generation cephalosporin	Cefepime	12	52.2%
Carbapenems	Meropenem	17	73.9%
	Imipenem	16	69.6%
β -lactam	Piperacillin/tazobactam	10	43.4%
	Cefepime/sulbactam	10	43.4%

TABLE 6 Comparison of costs, advantages, and disadvantages of RAA, conventional PCR, and real-time PCR.

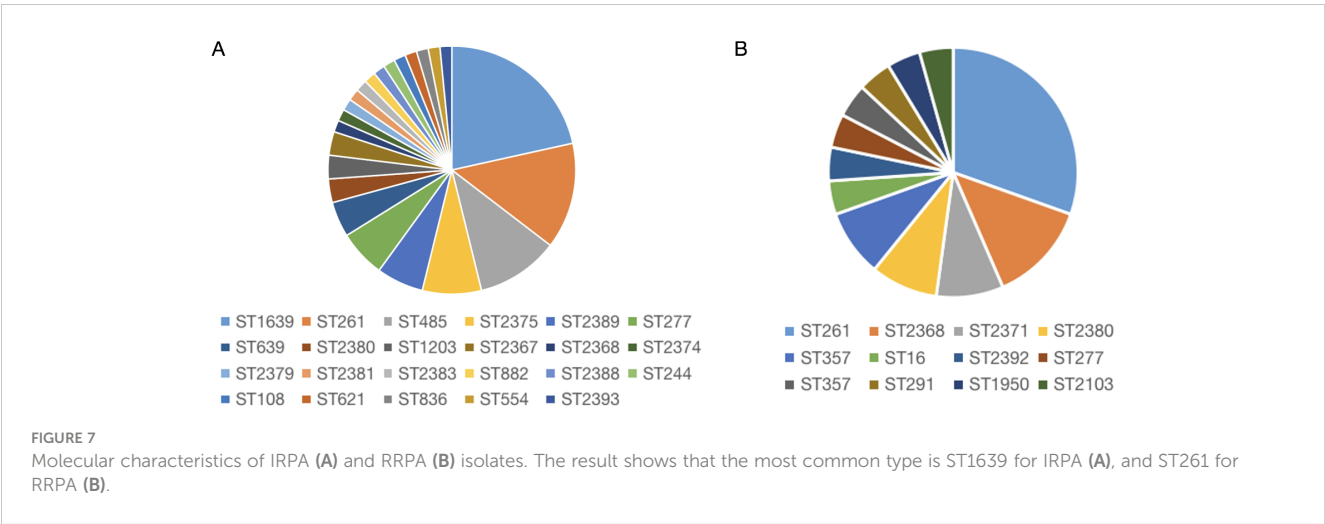
		RAA	PCR	Real-time PCR
Cost	Equipment	Low	High	High
	Reagent	High	Low	High
	Time	Very short (20 minutes)	1-3 hours	1-2 hours
Sensitivity		High (10 copies/reaction)	1000-10000 copies/reaction	100 copies/reaction
Advantages		Fast, high sensitivity, isothermal amplification, simple equipment requirements, on-site testing	Widely used, classic methods, complete international and domestic standards	Strong specificity Mature technology
Disadvantages		The reaction system contains proteases	Cumbersome operation, Time-consuming	Time-consuming, a highly sophisticated thermal cycler
Range of application		Suitable for primary hospitals and rapid diagnostic platforms with fast, on-site testing, and limited resources	Suitable for standardized and widely used nucleic acid testing.	Suitable for experimental research and clinical diagnosis

arr genes are located on the integron (Da Fonseca et al., 2008; Firoozeh et al., 2023). However, there is a certain discrepancy between the RAA detection results of *oprD* and the drug sensitivity results. Upon comparing the results with drug sensitivity tests, the concordance rate between RAA-IRPA detection results and drug resistance is 80%, surpassing the detection rate of the drug sensitivity tests. This is attributed to the tendency of *oprD* gene to undergo mutations (Quale et al., 2006; Kao et al., 2016; Do Rego and Timsit, 2023). It was discovered that this is caused by mutations, such as membrane topology and site-specific mutagenesis of *P. aeruginosa* porin OprD (Huang et al., 1995; Kim et al., 2016). In the deduced topological structure of the OprD protein, Li et al. identified external loops 2 and 3 as the entrance for basic amino acids and the binding site for imipenem. Furthermore, any substitution or deletion within loops 2 and 3 that results in a conformational change can cause imipenem resistance (Li et al., 2012). Ochs et al. found that deletions of amino acids 74-81, 84-91, 80-87, or 94-101 in loop L2, or amino acids 156-163 in loop L3, resulted in reduced sensitivity to imipenem compared to the wild type. This suggests that these amino acids play an

important role in imipenem binding or transmembrane transfer (Ochs et al., 2000). Overall, OprD is a well-characterized imipenem influx channel, and mutations in *oprD* render *P. aeruginosa* resistant to imipenem, posing significant challenges in clinical practice. These show that for some antibiotic resistances, detecting only one gene may be inaccurate and that all factors need to be considered to get more accurate results.

The establishment of RAA assay significantly saves time, cost, and manpower. However, rapid detection of resistance genes is still limited, and although RAA maintains its sensitivity and specificity, it may be impacted by the propensity for mutation in resistance genes, changes in drug resistance caused by mutated genes require careful consideration. Antimicrobial susceptibility testing, whole-genome sequencing, and other technologies can also serve as alternative methods. Additionally, the results of our experiments need validation from larger clinical samples to enhance the reliability of the findings.

In order to analyze the molecular characteristics of these drug-resistant strains, we performed MLST typing, virulence gene analysis, and antimicrobial susceptibility testing. Molecular



epidemiological methods were employed to analyze virulence genes, antibiotic resistance genes, and MLST (Curran et al., 2004; Huang et al., 2019; Blanc et al., 2020). MLST holds profound significance in preventing and controlling drug resistance (Maiden et al., 1998; Waters et al., 2012; Castaneda-Montes et al., 2018). Recently, this method has been applied in molecular studies of environmental microbiota and eukaryotic organisms (Chan et al., 2001; Cooper and Feil, 2004). Our results indicate that ST-1639 is the predominant subtype in IRPA (presumably a specific context or population), but there is no significant association between the ST type and virulence genes. ST-261 is the predominant sequence type of RRPA, and no significant association between ST typing and virulence genes was observed.

P. aeruginosa adapts to adverse host environments by secreting multiple virulence factors, aiding in successful host infection and disease onset. Detection of these virulence factors is crucial for understanding bacterial pathogenic mechanisms and guiding clinical treatments. The T3SS is a crucial secretion system that can inject various virulence factors into host cells, facilitating bacterial infection and pathogenesis (Soscia et al., 2007). Our results show a remarkably high detection rate for *exoS*, *exoT*, and *exoY*, indicating that these virulence factors play a driving role in the multidrug resistance of clinical *P. aeruginosa* strains. It is noteworthy that the detection rate of the *exoU* gene, a phospholipase with strong cytotoxicity, is extremely low in all clinical strains. Consistent with previous reports, this gene is almost always mutually exclusive to *exoS* (Juan et al., 2017; Zhao et al., 2023). The *exoU* and *exoS* genes encode distinct exotoxins, which have different pathogenic mechanisms and host effects. The selective expression of these exotoxins helps avoid competition for resources, allowing *P. aeruginosa* to efficiently utilize resources in specific environments. This mechanism also increases its ability to survive and reproduce in its host (Shaver and Hauser, 2004; Horna et al., 2019).

In summary, we established an RAA assay method for detecting IRPA and RRPA and evaluated its performance through sensitivity detection, specificity detection, and clinical sample detection. The results demonstrated that this method has high sensitivity and excellent detection performance. RAA assay does not rely on expensive equipment or specialized technicians, it is suitable for diagnostic laboratories with limited resources. This method is beneficial for future clinical treatment and disease control in primary hospitals. Therefore, RAA is an excellent tool for epidemiological surveillance.

For epidemiological analysis, we employed MLST, virulence gene identification, and Antimicrobial Susceptibility Testing. MLST analysis revealed that ST-1639 is the most common type of IRPA, while ST-261 is the most common type of RRPA. All tested strains possessed five or more virulence genes, indicating that the presence of these genes and their relationship to the severity of infections in patients warrants further attention. These analyses provide valuable guidance for clinical diagnosis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed from the corresponding author upon reasonable request.

Ethics statement

The studies involving humans were approved by Northwest University Experimental Animal Care and Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YZ: Formal analysis, Writing – original draft, Writing – review & editing. RS: Writing – review & editing. LM: Resources, Conceptualization, Writing – review & editing. LT: Methodology, Software, Supervision, Writing – review & editing. MZ: Project administration, Validation, Writing – review & editing. WL: Methodology, Visualization, Writing – review & editing. YC: Conceptualization, Data curation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1428827/full#supplementary-material>

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Genetic characterization of *Neisseria meningitidis* isolates recovered from patients with invasive meningococcal disease in Lithuania

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Introduction: *Neisseria meningitidis* is a gram-negative bacterium responsible for life-threatening invasive infections known as invasive meningococcal disease and is associated with high fatality rates and serious lifelong disabilities among survivors.

Methods: This study aimed to characterize *N. meningitidis* isolates cultured from blood and cerebrospinal fluid collected between 2009 and 2021 in Lithuania, assess their genomic relationships with European strains, and evaluate the possibility of using a cost-effective method for strain characterization, thus improving the national molecular surveillance of invasive meningococcal disease. In total, 321 *N. meningitidis* isolates were collected and analyzed using multilocus restriction typing (MLRT). Amplification of the *penA* gene and restriction fragment length polymorphism analysis were performed to identify the modified *penA* genes. Based on the MLRT genotyping results, we selected 10 strains for additional analysis using whole-genome sequencing. The sequenced genomes were incorporated into a dataset of publicly available *N. meningitidis* genomes to evaluate genomic diversity and establish phylogenetic relationships within the Lithuanian and European circulating strains.

Results: We identified 83 different strains using MLRT genotyping. Genomic diversity of *N. meningitidis* genomes analysed revealed 21 different sequence types (STs) circulating in Lithuania. Among these, ST34 was the most prevalent. Notably, three isolates displayed unique combinations of seven housekeeping genes and were identified as novel STs: ST16969, ST16901, and ST16959. The analyzed strains were found to possess virulence factors not commonly found in *N. meningitidis*. Six distinct *penA* profiles were identified, each with different frequencies. In the present study, we also identified *N. meningitidis* strains with new *penA*, NEIS0123, NEIS1320, NEIS1525, NEIS1600, and NEIS1753 loci variants. In our study, using the cgMLST scheme, Minimum Spanning Tree (MST) analysis

did not identify significant geographic relationships between Lithuanian *N. meningitidis* isolates and strains from Europe.

Discussion: Discussion: To our knowledge, this is the first study to employ whole genome sequencing (WGS) method for a comprehensive genetic characterization of invasive *N. meningitidis* isolates from Lithuania. This approach provides a more detailed and precise analysis of genomic relationships and diversity compared to prior studies relying on traditional molecular typing methods and antigen analysis.

KEYWORDS

N. meningitidis, antimicrobial resistance, virulence, pathogenicity, whole-genome sequencing, genotyping

1 Introduction

Neisseria meningitidis, a prevalent gram-negative bacterial commensal in the human upper respiratory tract, is also capable of causing large epidemics of invasive meningococcal disease (IMD) (Stephens et al., 2007). *N. meningitidis*, which asymptotically colonizes the nasopharynx in approximately 10% of healthy individuals, remains the main cause of meningitis and fulminant sepsis worldwide (Tzeng and Stephens, 2021). The transition from asymptomatic to invasive disease remains unclear. However, certain factors, such as the genetic and capsular structures of pathogenic strains, are thought to play a significant role (Mullally et al., 2021).

The distribution of serogroups of *N. meningitidis* shows significant variation worldwide, with six capsular types (A, B, C, W, X, and Y) responsible for most meningococcal disease cases (Lu et al., 2019). Although the capsule is the main factor determining the virulence of *N. meningitidis*, comparative genomic studies have revealed the presence of additional genomic factors in various lineages (Caugant and Maiden, 2009). Natural competence for genetic transformation and homologous recombination contribute to the extensive genomic diversity of meningococcal lineages, with recombination being the principal mechanism for introducing new genetic information (Schoen et al., 2009). Numerous factors have been linked to the pathogenesis of *N. meningitidis*, including increased invasiveness, overcoming host defenses, and resistance to antimicrobial treatment. Therefore, a comprehensive understanding of the adaptive and pathogenic mechanisms of *N. meningitidis* is important (Lu et al., 2019).

Molecular profiling of *N. meningitidis* is vital for global control of the disease. Genomic surveillance of bacterial meningitis pathogens facilitates the detection of emerging and spreading strains, leading to public health interventions (Rodgers et al., 2020). Over the past 10 years, in many European countries, epidemiological surveillance of *N. meningitidis* has been based on whole-genome sequencing (WGS) methods. The accessibility of WGS provides an opportunity to understand the biology and diversity of *N. meningitidis* (Bratcher et al., 2014). However, while WGS is employed in countries with the

necessary capacity and resources, in middle- or low-income countries, not only is the WGS methodology not in use, but simpler genotyping techniques, such as multilocus sequence typing (MLST), are not applied. Typically, methods with low discriminatory power, such as slide agglutination serogrouping or polymerase chain reaction (PCR)-based techniques for serogroup prediction, are used for epidemiological surveillance of infectious diseases in Lithuania. Importantly, at the national level for routine surveillance of meningococcal disease, comprehensive typing methods like MLST, considered a principal method for detailed molecular characterization, are not routinely employed. MLST analysis is typically conducted retrospectively by research groups (Sereikaitė et al., 2023; Ivaškevičienė et al., 2023) rather than as part of standard surveillance protocols. This does not ensure proper identification and typing of pathogens isolated during outbreaks/epidemics, transmission dynamics, risk factor identification, pathogenesis, and the etiologic attribution of pathogens; thus, it is not known whether isolates causing invasive diseases belong to the same virulent strain or if they are different strains (Eybpoosh et al., 2017).

The epidemiology of IMD varies geographically and over time (Tzeng and Stephens, 2021) and is influenced by meningococcal vaccines and isolation measures employed during the COVID-19 pandemic in 2020–2021 (Alderson et al., 2022).

Compared to other European countries, the epidemiological situation in Lithuania has been challenging for several years. The number of IMD cases reported in Lithuania from 2009 to 2020 (median, 1.65 per 100,000 inhabitants) was higher than that reported in other European countries (median, 0.63 per 100,000 inhabitants). During the period from 2009 to 2012, the average notification rate of IMD cases was 1.47 cases per 100,000 population. From 2013 to 2017, the average rate increased to 2.20 cases per 100,000 population. Although 2018 showed a significant decrease in the number of notified IMD cases (1.10 cases per 100,000 population in 2017), it was marked by the highest case fatality rate in the past 10 years, reaching as high as 21.74%. A relatively high case fatality rate persisted in 2019 at 18.75%. The

lowest number of notified IMD cases (0.25–0.39 per 100,000 population) and no associated fatalities were observed in 2020 and 2021 (European Centre for Disease Prevention and Control, 2023). Therefore, it is critically important to investigate the genotypic relationship of Lithuanian isolates with strains isolated in Europe to gain insights that can inform effective public health interventions.

2 Methods

2.1 Source of isolates

In total, 321 strains of *N. meningitidis* acquired from the National Public Health Surveillance Laboratory in Lithuania were examined. The isolates were collected from patients diagnosed with IMD between October 12, 2009, and August 25, 2021. Among them, 207 (64.5%) were isolated from blood and 114 (35.5%) from cerebrospinal fluid. Pure *N. meningitidis* cultures were stored at -80°C in tryptone soy broth containing 20% horse blood serum and 15% glycerol. To recover the isolates from long-term storage, 10 µL of freezing medium was transferred to Mueller–Hinton chocolate agar plates, and the isolates were incubated on the plates at 37°C with humidity for 18–24 h in an atmosphere of 5% CO₂. A single colony from a visually pure culture of each *N. meningitidis* isolate was selected for subsequent analysis.

2.2 Antibiotic susceptibility testing

Minimum inhibitory concentrations (MIC) for penicillin G, cefotaxime, rifampicin, and ciprofloxacin were determined using Liofilchem[®] MIC gradient strips (Liofilchem S.r.l., Italy) and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v13.1 (The European Committee on Antimicrobial Susceptibility Testing, 2023). Antimicrobial susceptibility testing was performed on Mueller–Hinton agar supplemented with 5% defibrinated sheep blood (Liofilchem S.r.l., Italy) and incubated at 35–37°C in 5% CO₂. For quality control, *Streptococcus pneumoniae* ATCC 49619 and *Escherichia coli* ATCC 25922 were used.

To compare the MIC values between the different groups, the Kruskal–Wallis test was applied. The data obtained were analyzed using IBM SPSS statistics software (version 21), and statistical significance was set at $p < 0.05$.

2.3 Genomic DNA extraction

The total DNA of *N. meningitidis* strains was extracted from suspended single bacterial colonies using a GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc.), following the manufacturer's protocol. DNA quality was assessed by measuring absorbance at 260/280 nm, and integrity was verified using 1% agarose gel electrophoresis.

2.4 Characterization of isolates

To confirm the identity of *N. meningitidis* and determine its serogroup, all isolates underwent PCR analysis. Meningococcus-specific genes (*porA* and *ctrA*) and genes specific to the serogroup (*sacD*, *siaD*, *cap29EH*, *wnmB*, *capZC*, *lcbB*, *xcbA*, *wnmA*, *synG*, and *galE/lipA*) were targeted as previously described (Zhu et al., 2012). Amplification products were separated on a 1% agarose gel. Genogroups for all publicly available *N. meningitidis* genomes from Lithuania were extracted from the pubMLST database.

All *N. meningitidis* strains were analyzed using multilocus restriction typing (MLRT), which was performed as described by Bennett et al (Bennett and Cafferkey, 2003). MLRT involves the restriction fragment length polymorphism analysis of PCR products obtained from seven housekeeping gene loci used in MLST (Maiden et al., 1998). PCR products were digested with *MspI* and *MnII* (Thermo Fisher Scientific Inc.) restriction endonucleases according to the manufacturer's instructions and analyzed by 1% agarose gel electrophoresis. Alleles at each of the seven loci were combined to obtain an allelic profile or restriction type (RT). All RT were manually scored and analyzed. A dendrogram was constructed using Hierarchical Clustering—Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) (Garcia-Vallvé et al., 1999) and visualized using interactive Tree of Life (iTOL) (Letunic and Bork, 2021).

Amplification of the *penA* gene and restriction fragment length polymorphism (RFLP) analysis were performed to identify the modified *penA* genes, following the method described by Antignac et al (Antignac et al., 2001). The *penA* PCR products were treated with restriction endonuclease *TaqI* (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and assessed by 1% agarose gel electrophoresis. To evaluate the relationship between *penA* RFLP and *penA* sequencing methods, we analyzed data of publicly available isolates from Lithuania in the PubMLST database (Jolley et al., 2018). Additionally, we used a BLASTn search against the NCBI nr database to detect homology between rarely detected *penA* alleles and *penA* genes of other *Neisseria* species. To identify *penA* sequence differences between strains and mutations associated with penicillin resistance, we conducted a multiple sequence alignment using Jalview (version 2.11.3.3) (Waterhouse et al., 2009), employing MAFFT with default settings. Based on the MLRT genotyping results, we selected 10 distinct strains for additional analysis using WGS (Table 1). The dendrogram representing genetic variation among 321 isolates of *N. meningitidis* based on MLRT analysis was instrumental in helping us objectively select different strains. Sub-clustering had a major impact on the strain selection process. First, we chose at least two strains (one isolated from blood and one from CSF) from each of the three largest sub-clusters. Secondly, we selected at least two distinct serogroups from each sub-cluster. Additionally, we included rarely identified serogroups (Y, E, NG). We did not choose strains from cluster 1 because we assumed the risk that these strains were from patients with weakened immune systems (patient age >64 years). Additionally, isolates from sub-cluster 3 were recently analyzed by other scientists in Lithuania

TABLE 1 Characteristics of the 10 selected *Neisseria meningitidis* isolates for whole genome sequencing analysis.

pubMLST ID	Year	Source	Patient age range	Genogroup	MLRT sub-cluster	RT (MLRT)
120132	2014	Blood	45-64	C	1	19
120136	2016	Cerebrospinal fluid	25-44	B	1	49
120133	2015	Blood	15-24	B	2	27
120134	2016	Blood	25-44	B	2	43
120135	2016	Blood	15-24	E	2	45
120139	2017	Cerebrospinal fluid	15-24	Y	2	76
120140	2021	Blood	45-64	B	2	83
119504	2011	Cerebrospinal fluid	45-64	B	4	1
120137	2016	Blood	15-24	NG	4	50
120138	2017	Blood	45-64	B	4	58

(Sereikaitė et al., 2023). We did not specifically select for specific restriction types. However, almost all the selected strains had unique restriction types (frequency of 1), with only one strain selected from the most commonly identified restriction type, RT1.

2.5 Bioinformatic analyses

The extracted DNA was sent to Novogene services (<https://www.novogene.com/eu-en>) for whole genome sequencing using the Illumina NovaSeq 6000 sequencing system and subsequent genome assembly. A paired-end sequencing strategy was used to sequence the samples. The coding genes were predicted using Augustus software (version 2.7) with homologous evidence. The scaffolds were subjected to RAST annotation server (Overbeek et al., 2014). Draft genomes of the described *N. meningitidis* strains were submitted to the PubMLST database (Jolley et al., 2018) with the following accession numbers: 119504, 120132, 120133, 120134, 120135, 120136, 120137, 120138, 120139, and 120140.

Phylogenetic networks were created for the invasive isolates of *N. meningitidis* from Lithuania (n = 43), which were the only genomes available in the PubMLST (Jolley et al., 2018) database until October 9, 2023. Of these, 10 genomes were sequenced in our study, and 33 genomes were submitted earlier. iTOL (Letunic and Bork, 2021) was used to construct neighbor-joining trees using concatenated nucleotides of the core-genome MLST (cgMLST) loci. Loci with paralogous hits within isolates were excluded from analysis.

A minimum spanning tree (MST) was created for a collection of 9490 *N. meningitidis* isolates using the GrapeTree tool on the PubMLST website (Jolley et al., 2018). The tree was constructed based on a cgMLST v2 scheme and was color-coded according to the country of isolation. The analysis specifically included invasive isolates collected in Europe.

tRNA, rRNA, sRNAs, Interspersed Repeat, and Tandem Repeat were predicted by Novogene.

The predicted protein-coding genes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) using

BLASTKOALA and the KEGG Mapper Reconstruction web server (Kanehisa et al., 2016).

The subcellular localization of the proteins was predicted using PSORTb version 3.0 (Yu et al., 2010). Putative adhesion domains in the encoded amino acid sequences were determined using the Pfam database (Mistry et al., 2021). We conducted a literature review to identify experimentally validated bacterial adhesins, such as performing a keyword search for “adhesin,” “adherence,” “adhesion,” “adhesive,” “chitin binding,” “collagen binding,” “laminin binding,” “mucin binding,” “membrane adhesin,” “*Neisseria* adhesin,” “pilin,” “pilus,” “autotransporter,” “polysaccharide capsule,” “porin,” and “two-partner secretion” in the InterPro 96.0 database (Paysan-Lafosse et al., 2023).

To verify the pathogenicity and virulence factors, PathogenFinder v1.1 (Cosentino et al., 2013) and the Virulence Factor Database (VFDB) (Liu et al., 2019) were used. Antimicrobial resistance genes were identified using Resistance Gene Identifier from CARD (Alcock et al., 2020), and alleles linked to reduced antibiotic susceptibility were detected using the genome comparator tool in PubMLST (Jolley et al., 2018).

3 Results

3.1 *N. meningitidis* serogrouping

The 321 *N. meningitidis* isolates were serogrouped, and the distribution of serogroups determined by PCR methods was as follows: 292 (91.0%) were serogroup B, 23 (7.2%) were serogroup C, 3 (0.9%) were serogroup Y, 1 (0.3%) was serogroup W135, 1 (0.3%) was serogroup E, and 1 (0.3%) was non-groupable (Supplementary Table S1).

3.2 Antibiotic susceptibility testing

All the *N. meningitidis* isolates were susceptible to penicillin, cefotaxime, rifampicin, and ciprofloxacin (Supplementary Table S1).

3.3 *N. meningitidis* genetic diversity

3.3.1 MLRT analysis

Several restriction patterns (alleles) were observed for each of the seven loci examined, indicating polymorphisms at these loci (Figure 1). The number of alleles varied between four in *adk*, the

most conserved locus, and 14 in *pgm*, suggesting different evolution rates for different loci.

Of the 321 strains examined, 83 different RTs were identified; their interrelationships are illustrated in the dendrogram (Figure 2). The UPGMA dendrogram illustrated genetic variation among the isolates, categorizing them into three major clusters. Cluster 1 consisted of only

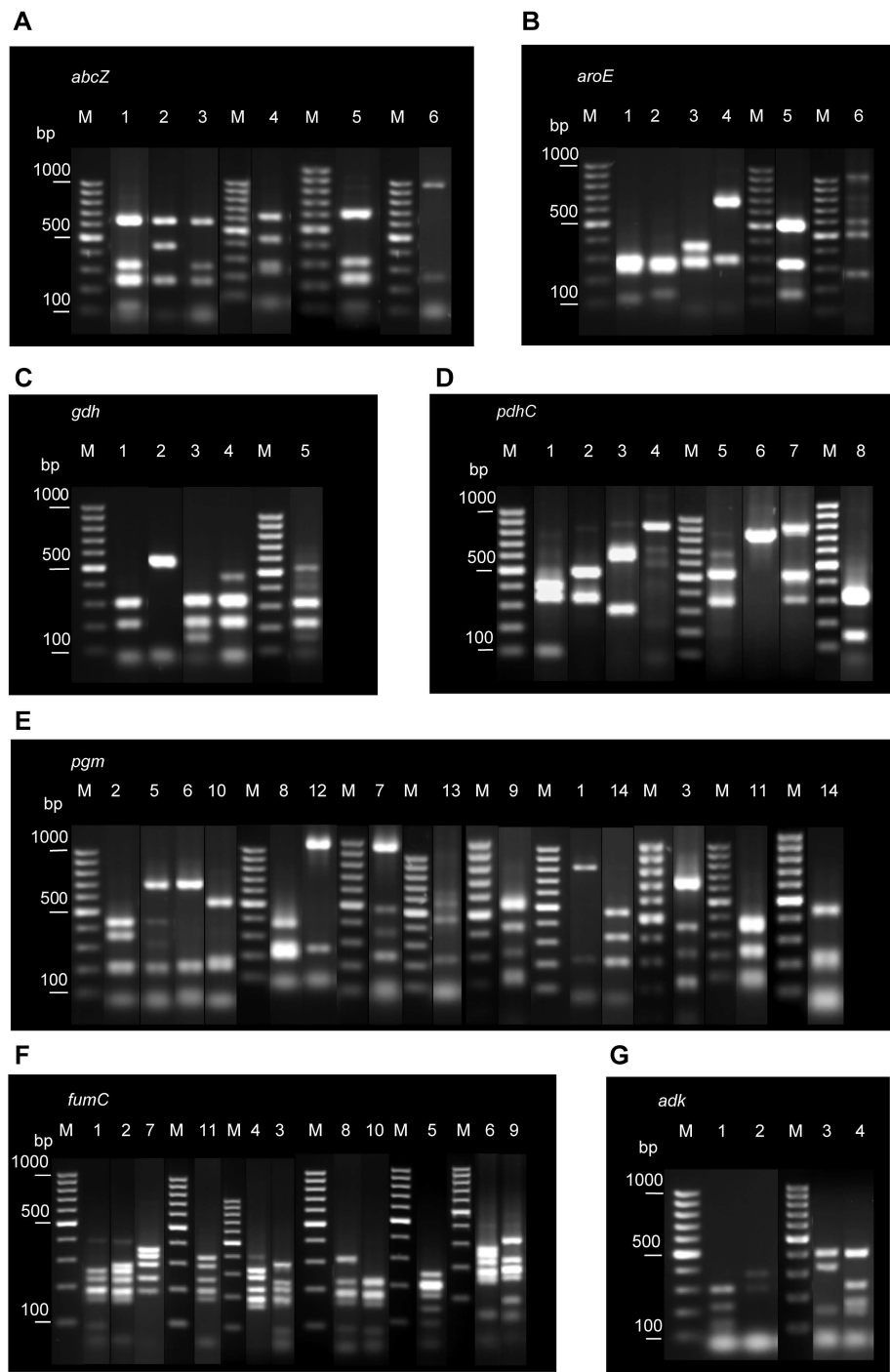
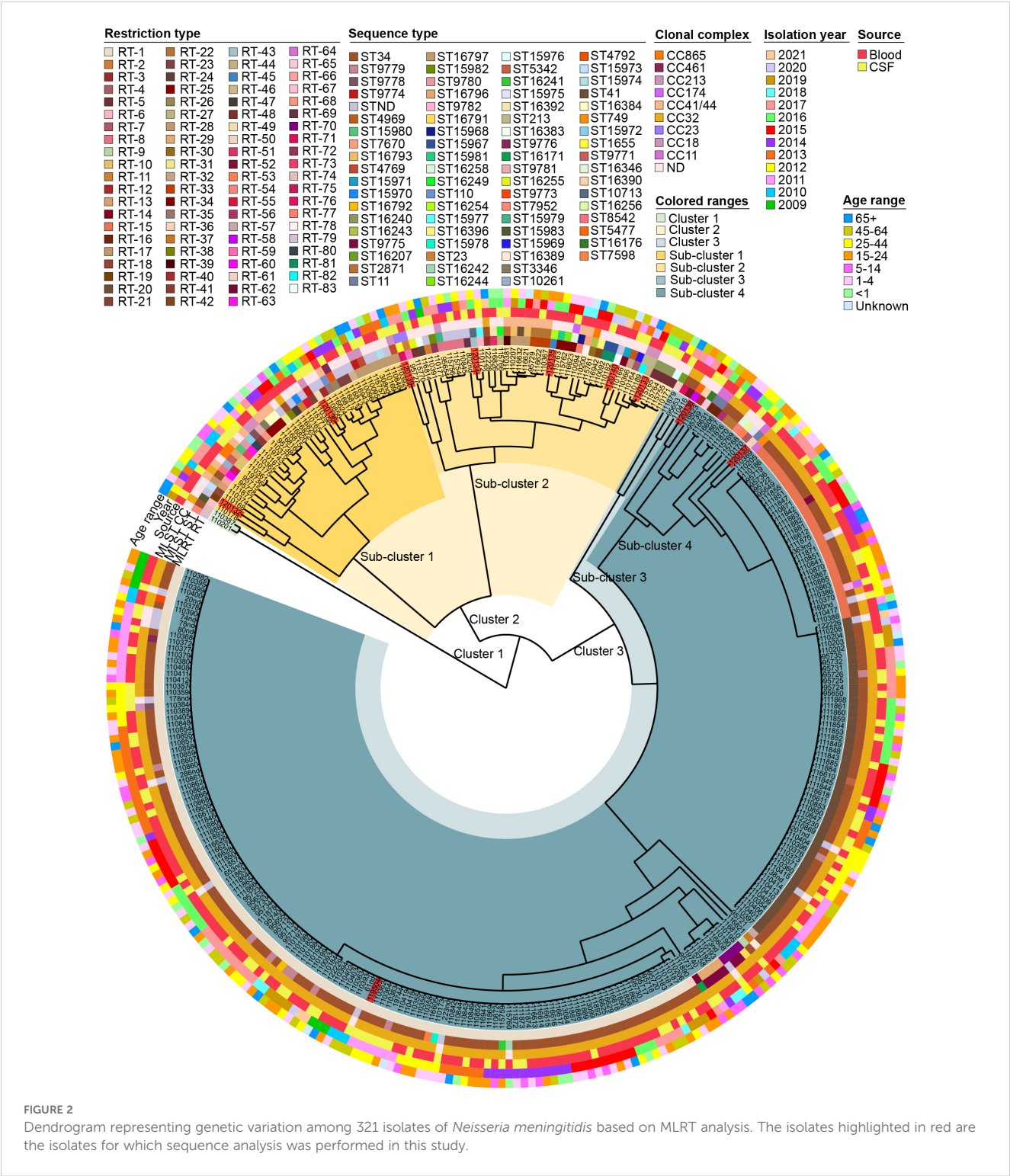


FIGURE 1 Examples of different restriction patterns obtained following *MspI* or *MnlI* digestion of PCR-amplified regions of the seven housekeeping genes analyzed: *abcZ* (A), *aroE* (B), *gdh* (C), *pdhC* (D), *pgm* (E), *fumC* (F), and *adk* (G). Lane numbers correspond to allele codes. Lane M, GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific).



two isolates. Clusters 2 and 3 were further divided into two subclusters. Subclusters 1, 2, 3, and 4 were again divided into groups. The first, second, third, and fourth groups included 4, 33, 4, and 39 isolates, respectively. The fifth group comprised only 1 isolate. The sixth group comprised 2 isolates, the seventh group consisted of 93 isolates, and the eighth group included the largest number of isolates (143). Phylogenetic analyses of the isolates revealed that each subcluster consisted of *N. meningitidis* isolates from various isolation years, sites, and patient age

groups (Figure 2). No significant patterns related to the isolation year, site, or patient age group could be inferred from this clustering. Based on the MLRT genotyping results, we chose 10 distinct strains for molecular characterization using WGS to elucidate evolutionary dynamics, assess regional prevalence, and classify strains more precisely, while also identifying genetic markers and inferring transmission pathways to gain comprehensive insights into emerging strains for enhanced public health understanding and preparedness. The dendrogram was

instrumental in helping us objectively select different strains. Sub-clustering had a major impact on the strain selection process, providing additional context and validation for our choices.

To evaluate the relationship between MLRT and MLST genotyping methods, we analyzed data from 10 sequenced isolates and 33 publicly available isolates from Lithuania in the pubMLST database. The distribution of RT, ST, and CC is outlined in the [Supplementary Table S2](#). We identified some discrepancies between the identified RTs and STs. The strains marked as RT1, RT15, and RT20 were identified as ST34 according to the MLST method. RT15 differs from RT1 by one allele in two loci, *adk* and *gdh*. RT20 differs from RT1 by one allele in the *gdh* locus.

Although there is some discrepancy between the identified RTs and STs, the clonal relationships among *N. meningitidis* strains are clearly delineated. In our MLRT and MLST comparison analysis, we identified that all strains marked as RT1, RT15, RT20, RT29, and RT70 belonged to the ST-32 complex. According to the MLRT genotyping method, all these strains have identical alleles in the *aroE*, *fumC*, *phdC*, and *pgm* loci. According to the MLST method, all strains belonging to the ST-32 complex have identical alleles in the *adk*, *fumC*, *phdC*, and *pgm* loci. It is noteworthy that during the visual assessment of electrophoresis, there were doubts in distinguishing allele 2 from allele 3 in the *adk* locus, which might be the cause of the discrepancies.

The strain marked as RT50 was associated with the ST-11 complex, while RT19 and RT49 belonged to the ST-18 complex. Strains marked as RT27 and RT82 were found to be part of the ST-213 complex, and RT76 belonged to the ST-23 complex. Additionally, RT28, RT77, and RT78 were classified within the ST-41/44 complex. Notably, the strain marked as RT17 was the only strain to belong to two distinct clonal complexes: ST-18 (n=2, 66.7%) and ST-32 (n=1, 33.3%).

Our analysis revealed that certain RTs belonged to specific clonal complexes, suggesting that MLRT can serve as a predictor for MLST clonal complexes in our dataset.

3.3.2 *penA* analysis

Amplification of the *penA* gene and RFLP analysis were performed to identify the modified *penA* genes. Amplification products were obtained for all analyzed strains. Subsequent digestion with *TaqI* and separation on a 1% agarose gel resulted in the identification of six distinct profiles ([Figure 3A](#)). A unique identifier was assigned to each profile and labeled patterns 1–6. Among them, *penA* 1 (71.88%, n = 230) was the most prevalent, followed by *penA* 2 (22.19%, n = 71). The distribution of different *penA* restriction patterns in the Lithuanian *N. meningitidis* strains is shown in [Figure 3B](#).

The penicillin MIC values in isolates with *penA* 1 (range: 0.016–0.250 mg/L, median = 0.125) were significantly higher than those in isolates with *penA* 2 (range: 0.016–0.190 mg/L, median = 0.023) and *penA* 4 (range: 0.016–0.047 mg/L, median = 0.023) patterns ($p < 0.001$) ([Supplementary Table S1](#)). None of the isolates analyzed were resistant to penicillin.

To evaluate whether the *penA* RFLP method used in this study could help identify different *penA* gene modifications, we compared the results with sequencing data from 10 isolates and included an additional 33 publicly available isolates from Lithuania in the pubMLST database. In total, 11 distinct *penA* gene alleles were identified. The *penA52* allele constituted the majority of the analyzed isolates (55.80%, n=24). Notably, all isolates with the *penA52* allele in our study were identified as having the *penA1* restriction pattern. The distribution of *penA* gene alleles and their comparison with the *penA* RFLP method results are presented in the [Supplementary Table S2](#).

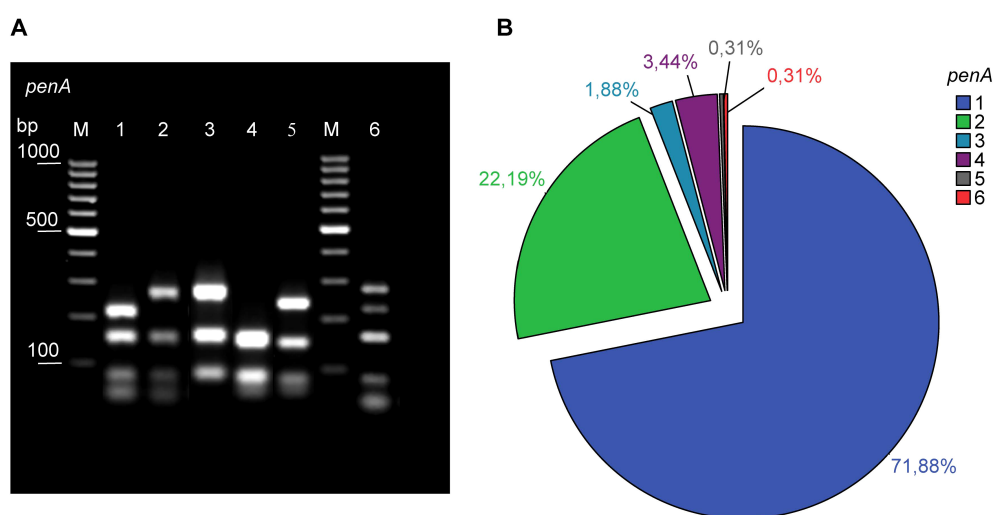


FIGURE 3

(A) Examples of distinct restriction patterns obtained after *TaqI* digestion of the PCR-amplified segment of the *penA* gene, which encodes penicillin-binding protein 2. Lane numbers are associated with allele codes. Lane M, GeneRuler 100 bp DNA Ladder. (B) Distribution of distinct restriction patterns obtained after *TaqI* digestion of the PCR-amplified segment of the *penA* gene.

3.3.3 cgMLST analysis

The genomic diversity of *N. meningitidis* in Lithuania was investigated by analyzing a set of 10 strains specifically examined in this study. To obtain a more thorough and detailed insight into genomic diversity, an additional 33 publicly available genomes collected between 2011 and 2021 were included. All genomes were derived from human sources and associated with invasive infections (Figure 4).

Our analysis revealed 21 sequence types (STs) circulating in Lithuania. The most prevalent was ST34 (76.19%, $n = 16$), followed by ST9775 (19.05%, $n = 4$) and ST9779 (14.29%, $n = 3$). Other STs, such as ST213 and ST15967, were detected in at least two genomes, whereas 16 different STs were observed in individual genomes.

Notably, three isolates (ID 120132, 120136, and 120140) displayed unique combinations of seven housekeeping genes and were designated as novel STs upon submission to the PubMLST database. Specifically, isolate 120132 had a novel ST16969 profile, isolate 120136 had an ST16901 profile, and isolate 120140 had an ST16959 profile. Isolate 120132 was obtained from the blood of a 47-year-old male, isolate 120136 from the cerebrospinal fluid (CSF) of a 40-year-old female, and isolate 120140 from the blood of a 46-year-old male.

3.3.4 Comparison of local genomic epidemiology with international genomic epidemiology using the cgMLST scheme

Incorporating all Lithuanian isolates into a dataset of publicly accessible *N. meningitidis* genomes allowed the evaluation of their genomic diversity and phylogenetic relationships with circulating strains in Europe. Currently, the dataset is predominantly composed of isolates from the United Kingdom ($n = 5726$), the Netherlands ($n = 684$), Germany ($n = 534$), and France ($n = 530$), contrasting with other European countries, several of which have fewer than 10 sequenced isolates as of the analysis date (e.g., Portugal, Latvia, and Cyprus). Using the cgMLST scheme, the MST of 9490 *N. meningitidis* strains from various European countries revealed no significant geographic relationships. A widespread dissemination of *N. meningitidis* strains was observed within the European region, with the root of the MST originating in Sweden and the United Kingdom. The examined isolates belonged to 43 clonal complexes, with the majority composed of the ST-11 complex (26.0%) and the ST-41/44 complex (16.7%). In Lithuania, however, the ST-32 complex was predominant (58.1%), contrasting with its lower prevalence (7.5%) in the broader European region. Out of the 9490 analyzed isolates, 814 (8.6%) were not assigned to a

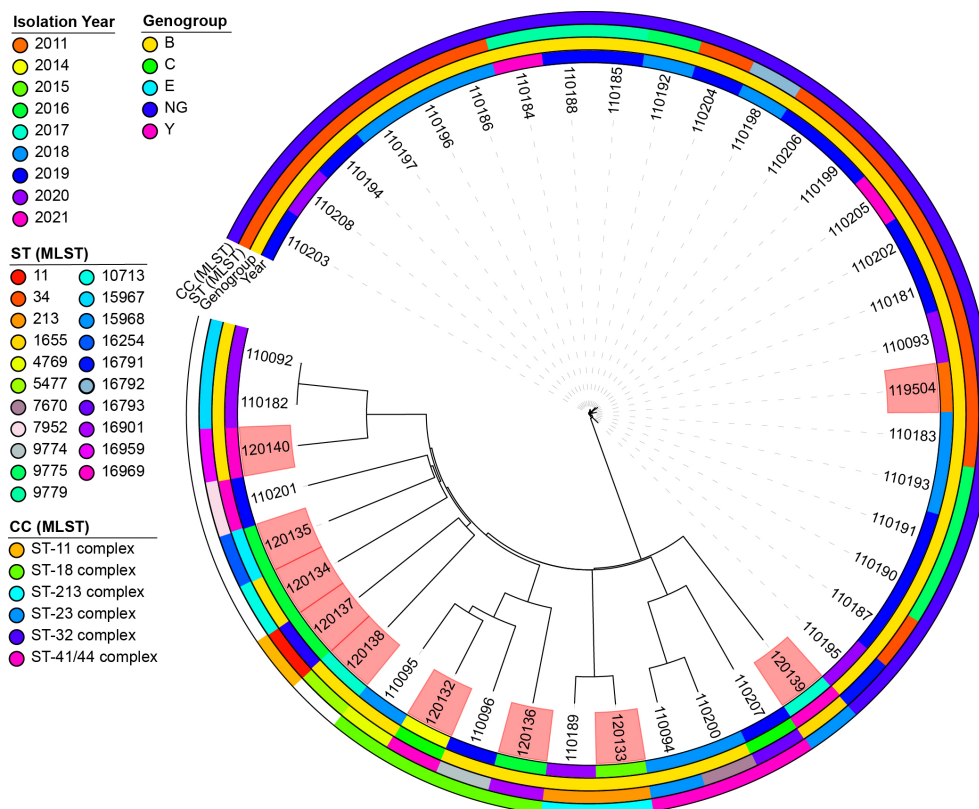


FIGURE 4

A neighbor-joining tree was constructed based on concatenated cgMLST allele sequences of 43 *Neisseria meningitidis* strains. The isolates are visually represented with color coding, indicating their genogroup, sequence type, clonal complex, and year of isolation. The phylogenetic analysis was performed using the iTOL plug-in available on the PubMLST website (<https://pubmlst.org/>). The scale bar on the tree represents the genetic distance between the isolates. The isolates highlighted in red are the isolates for which sequence analysis was performed in this study. NG, non-groupable; ST, sequence type; CC, clonal complex.

clonal complex. Of the 10 Lithuanian isolates analyzed in this study, eight belonged to different clades of relatedness, while only two strains (ID 120132 and 120136) were closely related (Figure 5).

MST demonstrated that the Lithuanian ST-32 complex isolates ($n = 25$), including the isolate analyzed in this study (ID 119504), were genetically related and formed a single clade, along with isolates from various European countries. The newly identified STs, ST16969 (ID 120132) and ST16901 (ID 120136), were

closely related to an isolate found in England in 2016 (ID 53173) and belongs to ST18. Additionally, several isolates from Lithuania exhibited close genetic relationships with isolates from England, Northern Ireland, Poland, and the Czech Republic, indicating potential transmission events and genetic exchange across borders. Supplementary Figures S1, S2 illustrate the genetic relationships between *N. meningitidis* strains from Lithuania and those circulating in Europe.

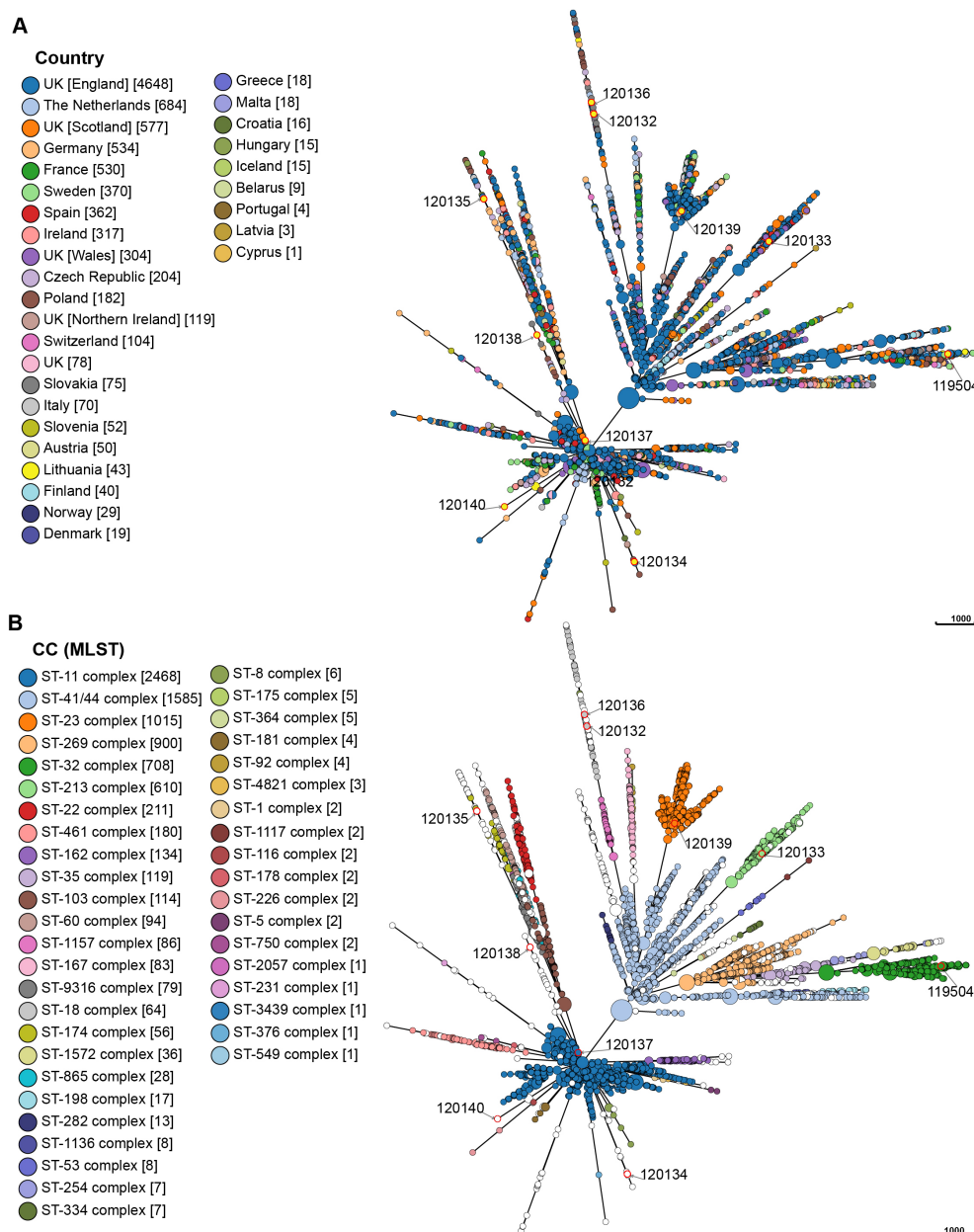


FIGURE 5

The minimum spanning tree based on the results of cgMLST. Each circle represents genetically related isolates. The length of branches between each node represents the number of different alleles (out of cgMLST genes) that differ between two linked nodes. The size of each circle is proportional to the number of strains it contains. The colors indicate either the corresponding countries of isolation (A) or the clonal complex (MLST) (B). The isolates marked with a red circle are the isolates for which sequence analysis was performed in this study. The pubMLST ID number for each isolate for which sequence analysis was performed in this study is written next to each circle.

3.4 Analysis of WGS data

In this study, ten draft genomes of *N. meningitidis* strains were used. The characteristics of the assembled genome are listed in [Supplementary Table S3](#). The number of scaffolds per genome assembly varied from 70 to 121, and the N50 values were between 29,431 and 55,311 base reads. The average genome size of the 10 strains was 2,163,040 bp, ranging from 2,060,116 bp (ID 120137) to 2,283,261 bp (ID 120136). The average GC content of all the genomes was 51.66%, ranging from 51.46% (ID 120138) to 51.95% (ID 120137).

The average number of coding genes predicted by Augustus (version 2.7) was 2178, ranging from 2082 (ID 120137) to 2305 (ID 120136). In contrast, the average number of CDSs by RAST was 2567, ranging from 2429 (ID 120137) to 2729 (ID 120136). Only 28–31% of CDSs in each strain could be functionally categorized into subsystems. In all studied strains, subsystems such as protein metabolism, amino acids and derivatives, cofactors, vitamins, prosthetic groups, and pigments were found to have a higher number of functional genes. No genes associated with potassium metabolism, motility, or chemotaxis were detected in any strain. [Supplementary Table S3](#) and [Supplementary Figure S3](#) provide a comprehensive overview of the genome characteristics of *N. meningitidis* strains and their subsystem statistics.

Based on KEGG analysis, the CDSs were categorized into six sub-categories. The strains conserved 1264 (ID 120137) to 1288 (ID 120136) CDSs, which were further classified into 40 functional KEGG subcategories. It is worth mentioning that all strains exhibited a noticeably higher abundance of genes associated with metabolic pathways (CDSs 783–803), followed by genetic information processing (CDSs 183–186) and human diseases (CDSs 106–112) ([Supplementary Table S4](#)). Pathway modules ([Supplementary Table S5](#)) showed high similarity across strains, with few differences observed. The exclusive metabolic features detected in strains 120136, 120139, and 120140 were complete pathways for dTDP-L-rhamnose biosynthesis. In other strains, this pathway was either not detected (119504 and 120133) or was incomplete (120132, 120134, 120135, 120137, and 120138). Incomplete pathways for Shikimate and Siroheme biosynthesis were observed in strains 120134 and 120137, respectively.

3.4.1 Genetic factors encoding adhesion properties

Exploration of extracellular proteins or outer membrane-attached proteins encoded within the genomes of the analyzed strains, as indicated by PSORTb, revealed an average of 66 proteins (range: 62–77). Additionally, all genomes were examined for the presence of adhesion-related domains. We identified 50 distinct adhesion-related domains. The subsequent domains were found in all strains: PF03865 and PF13018. The following domains were exclusively detected in strain ID 120139: PF06122, PF05309, PF06834, and PF09673. The results of identifying adhesion-related domains and their frequencies in the analyzed genomes are presented, and a complete list of domains detected using the Pfam database can be found in [Supplementary Tables S6, S7](#).

[Supplementary Table S8](#) contains a comprehensive list of proteins found in the genomes of *N. meningitidis* strains, including those located extracellularly, attached to the outer membrane, and putative adhesins in other cellular locations, along with their respective domains, as identified using the Pfam database. From these analyses, we identified genes encoding putative adhesins or adhesion-related proteins. These candidates should be further investigated in future functional studies.

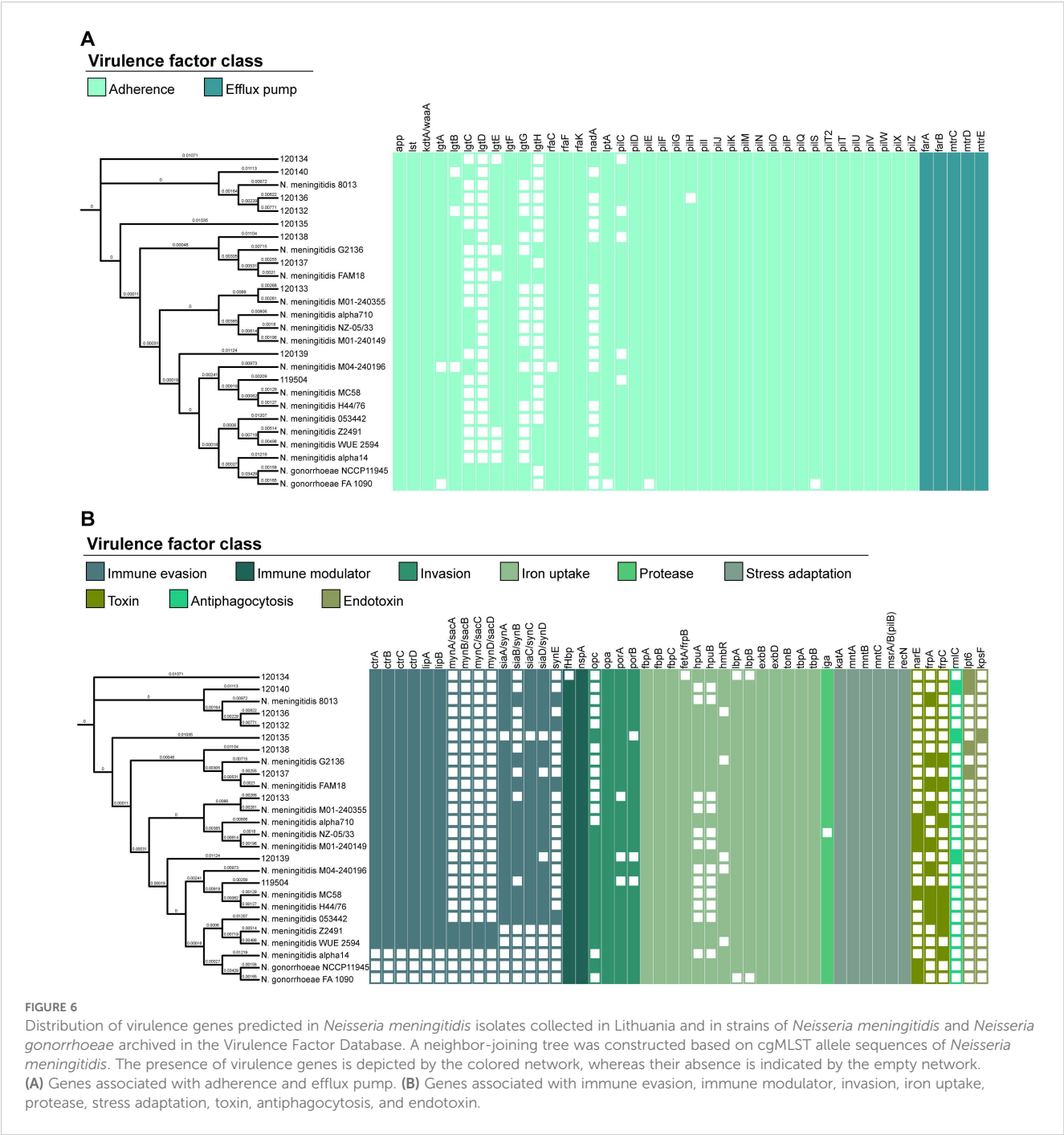
3.4.2 Genome-based assessment of the potential for pathogenicity

The 10 *N. meningitidis* genomes were screened for pathogenicity and virulence genes using the PathogenFinder server, scoring each strain as a potential human pathogen ([Supplementary Table S3](#)). Strain 120134 had the lowest pathogen score of 85.3%. A total of 1732 genes associated with pathogenicity were identified, with nonuniform distribution across the genomes of each strain. Strains 119504 and 120137 exhibited the highest number of detected pathogenicity determinants (656 and 636, respectively), while strain 120133 had the lowest (350). All 10 isolates were positive for 17 pathogenicity determinants, with five of them categorized as hypothetical proteins. The pathogenic determinants present in a single isolate were defined as unique determinants. In total, 873 unique pathogenicity determinants were identified, 383 of which were designated as hypothetical and 237 as putative proteins. Strain 120134 had the fewest unique determinants (29), while strains 119504 and 120137 had the most at 345 and 227, respectively.

The VFDB system confirmed the presence of various virulence genes, categorized into 11 classes, with most genes associated with adherence (46.3%) and iron uptake (17.1%) ([Figure 6](#)). Among the identified genes, 57 (69.5%) were present in all analyzed strains. Notably, genes related to antiphagocytosis (*rmlC*) and endotoxins (*lpt6* and *kpsF*) were exclusively found in Lithuanian isolates analyzed in this study. Isolates 120134 and 120135 lacked the *fetA/frpB* and *pilH* genes, respectively, in contrast to the other isolates in this study and the strains stored in the database. [Supplementary Table S9](#) provides a comprehensive overview of the virulence factor genes of the *N. meningitidis* strains analyzed in this study.

3.4.3 Antibiotic resistance

For all the strains examined, at least one gene or gene mutation leading to resistance to antimicrobial agents was identified. Eight antibiotic resistance genes have been detected that confer resistance to antibacterial free fatty acids, macrolides, cephalosporins, cephamycin, tetracycline, penicillin, disinfecting agents, and antiseptics. Overall, the most common resistance genes detected were *farB* (90.0%), which mediates resistance to long-chain antibacterial fatty acids, and *mtrA* (100%), a macrolide antibiotic. The mutation V57M in *rpsJ*, conferring tetracycline resistance, was observed in strain 120139. However, these factors are not advantageous because they are not the antibiotics of choice for the treatment of IMD. Three strains harbored mutations in *N. meningitidis* PBP2, conferring resistance to beta-lactam. A complete



list of resistance genes detected using the CARD database is provided in [Supplementary Table S10](#).

We identified allele diversity at all loci among the 11 antibiotic susceptibility genes analyzed using PubMLST. Three isolates harbored alterations in *penA*, encoding penicillin-binding protein 2, with specific substitutions: isolate ID 119504 had the *penA52* allele, isolate ID 120136 had *penA686*, and isolate ID 120138 had *penA1191*. In the PubMLST database, only two isolates were found to have the *penA686* allele: one from Lithuania and one from Italy (ID 40340), while the *penA1191* allele was identified exclusively in the isolates examined in this study. Next, we used BLASTn to detect homologies between rarely detected *penA* alleles (*penA686* and *penA1191*) and *penA* genes from other *Neisseria* species. A BLASTn search against the NCBI nr database yielded hits in other *Neisseria* species with high (>95%) sequence identity. One of the rarest alleles (*penA1191*) showed an identity score of 96.51% with the *penA* gene sequences of *N. gonorrhoeae* strain NGMAST ST7268 (GenBank accession no. KC192769.1) and *N. gonorrhoeae* strain 31188 (GenBank accession no. JF893455.1). In the *penA686* nucleotide sequence analysis, we found matches with 95.08% identity to the *N. gonorrhoeae* strain NG9901 *penA* gene (GenBank accession no. AB608050.1).

The β -lactamase gene (*blaROB-1*), associated with resistance against β -lactam antibiotics (Hong et al., 2018), was not detected in

TABLE 2 Allelic profiles of antibiotic resistance genes in *Neisseria meningitidis* isolates from Lithuania.

Locus	pubMLST ID									
	119504	120132	120133	120134	120135	120136	120137	120138	120139	120140
<i>gyrA</i>	2	4	3	4	12	11	4	4	1	3
NEIS0123	132	41	39	302	218	41	1	2909	39	2382
NEIS0414	4	1	22	244	168	207	1	1	45	X
NEIS1320	2	2216	97	1	174	2217	159	9	107	2218
NEIS1525	51	50	49	623	2155	2156	44	224	70	2627
NEIS1600	22	260	57	289	1570	1571	172	202	79	1572
NEIS1635	4	7	7	9	11	7	1	2	7	7
NEIS1753	189	2643	70	205	211	3498	59	2644	8	9
NEIS3240	X	X	X	X	X	X	X	X	X	X
<i>penA</i>	52	5	34	1	5	686	1	1191	22	4
<i>rpoB</i>	2	1	34	34	5	1	9	X	34	34

Each locus was assigned an allele number according to its DNA sequence using the PubMLST database.
X – missing alleles.

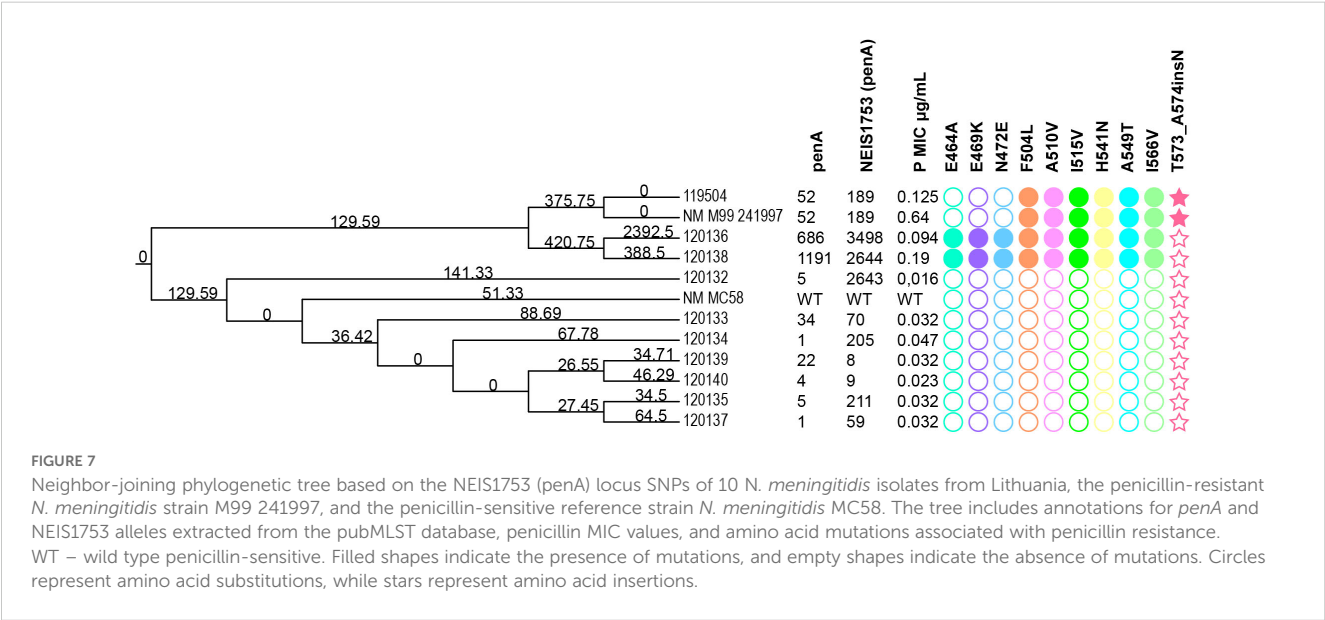
any analyzed isolates. No alleles linked to resistance to fluoroquinolones (*gyrA*, NEIS1320, NEIS1525, and NEIS1600) or rifampicin (NEIS0123 and *rpoB*) were found (Table 2).

3.4.3.1 Alterations of PBP2

The complete nucleotide sequences of the NEIS1753 (*penA*) locus from 10 analyzed isolates were examined to identify alterations in *penA* gene. These sequences were compared with the *N. meningitidis* MC58 strain (GenBank accession no. AE002098.2), which harbors the wild-type *penA* gene, and with the *N. meningitidis* M99 241997 strain extracted from pubMLST database (ID 89367) and which is resistant to penicillin (MIC=0.64 µg/mL) (Figure 7; Supplementary Figure S4). Three isolates harbored different mosaic *penA* alleles (*penA*52, *penA*686, and

*penA*1191). The sequences of the divergent mosaic alleles were 89.55% to 97.94% identical to the wild-type sequence represented by the MC58 strain. Specifically, isolate 119504 showed 97.94% identity, isolate 120136 showed 89.55% identity, and isolate 120138 showed 96.19% identity. This comparison was based on the complete nucleotide sequences of the NEIS1753 (*penA*) locus. Isolates 119504 and 120138 have slightly increased penicillin resistance (MICs of 0.125 µg/mL and 0.19 µg/mL, respectively).

In the *penA* C-terminal domain of PBP2 (amino acids 298 to 581), the amino acid sequences were aligned. There are 12 amino acid differences between NmMC58 and Lithuanian isolate ID 119504, 24 differences between NmMC58 and isolate ID 120136, and 14 differences between NmMC58 and isolate ID 120138 (Supplementary Figure S4). All three isolates possessed six



penicillin-resistance-associated mutations (F504L, A510V, I515V, H541N, A549T, and I566V). These mutations are also found in the penicillin-resistant strain M99 241997. Additionally, isolates ID 120136 and ID 120138 possessed the E464A, E469K, and N472E substitutions, which were not found in the M99 241997 strain. All these amino acid sites have been previously linked to decreased susceptibility to beta-lactam antibiotics.

Lithuanian isolate ID 119504 and the penicillin-resistant strain M99 241997 have an insertion of a single asparagine amino acid between Thr573 and Ala574 (T573_A574insN) along with a substitution of one amino acid downstream (A574V). The sequences of the *penA* genes of isolates ID 120132, 120133, 120134, 120135, 120137, 120139, and 120140 were identical to that of the *N. meningitidis* MC58 strain.

4 Discussion

This study aimed to characterize the isolates of *N. meningitidis* causing IMD collected in Lithuania from 2009 to 2021. Various techniques were used, including MLRT genotyping, *penA* gene RFLP analysis, phylogenetic group detection, and WGS analysis.

The epidemiology of IMD has undergone significant changes due to factors like vaccination efforts, environmental conditions, ease of travel, and mass gatherings (Acevedo et al., 2019). Although Europe has seen an increase in serogroup W meningococcus (MenW) and MenY cases (Zografaki et al., 2023), MenB remains the major cause of IMD in Lithuania, with no recent rise in MenW and MenY cases. A rapid decrease in the number of IMD cases was observed shortly after the initiation of the initial lockdown in numerous countries during the COVID-19 pandemic (Brueggemann et al., 2021). A decline in IMD cases was observed in Lithuania starting in 2018, before the COVID-19 pandemic, reflecting the impact of vaccination efforts, including the introduction of the 4CMenB vaccine (Bexsero) into the national immunization program in July 2018. In recent studies conducted in Lithuania, the MenDeVAR Index method (Rodrigues et al., 2021) was used to estimate the predicted coverage of the 4CMenB and MenB-FHbp vaccines. The findings concluded that both serogroup B vaccines demonstrate potential to protect against IMD in Lithuania (Sereikaitė et al., 2023). Importantly, this study analyzed isolates recovered in Lithuania from 2009 to 2019, including the period before vaccination and shortly after the implementation of the 4CMenB vaccine (Bexsero) into the national immunization program in July 2018. Therefore, it is crucial to determine the MenDeVAR index and conduct a comprehensive characterization of *N. meningitidis* in the post-vaccination period, which would provide valuable insights for public health strategies.

Studies suggest that diverse regional surveillance systems and the evolution of hypervirulent *N. meningitidis* strains pose a potential threat, potentially increasing IMD incidence (Acevedo et al., 2019). For effective meningococcal disease surveillance, high-resolution, comprehensive, and portable typing schemes are crucial (Jolley et al., 2007). In this study, MLRT method (Bennett and Cafferkey, 2003) was used to characterize *N. meningitidis* strains,

revealing polymorphisms at the examined loci. MLRT genotyping of 321 isolates from Lithuania identified 83 strains, highlighting MLRT's discriminatory, rapid, and cost-effective nature for *N. meningitidis* strain characterization. These findings support previous studies characterizing *N. meningitidis* strains (Bennett et al., 2020). The population of *N. meningitidis* isolates in Lithuania displayed heterogeneity, consistent with global isolate diversity trends (Caugant and Brynildsrud, 2019). Our results indicate that MLRT method can assist in identifying clonal relationships among *N. meningitidis* strains associated with meningococcal disease, without the need for expensive equipment or a high level of technical expertise to perform. This simple and cost-effective method can be used as a screening tool to quickly identify newly emerging *N. meningitidis* strains. It helps in objectively selecting new strains for further analysis using more sensitive techniques like whole-genome sequencing. This approach is particularly useful in countries where advanced methods like MLST genotyping or WGS are not routinely used for epidemiological surveillance.

Treatment options for confirmed IMD typically involve third-generation cephalosporins, penicillin when the strain is susceptible, or chloramphenicol for beta-lactam antibiotic allergies (Nadel, 2016). Recently, strains of *N. meningitidis* resistant to antibiotics recommended for treatment and chemoprophylaxis have been detected globally (Rostamian et al., 2022). Modifications to penicillin-binding protein 2 reduce its affinity for penicillin, resulting in decreased susceptibility (Acevedo et al., 2019). In this study, we identified six distinct *penA* profiles using amplification of the *penA* gene and RFLP analysis, providing a cost-effective method for investigating prevalent *penA* modifications.

In previous research, geographic variation in the distribution of different *penA* alleles has been observed (Taha et al., 2007). In 2016, *N. meningitidis* serogroup B ST-32 complex, which harbored the *penA52* allele, was associated with a clonal epidemic of IMD in France (Thabuis et al., 2018). Isolates in Lithuania predominantly harbored the *penA52* allele, which this study unequivocally assigned to the *penA1* restriction pattern. We found that isolates with the *penA1* restriction pattern have a statistically significantly higher penicillin G MIC. Other studies have also observed higher penicillin MIC in strains with the *penA52* allele (Hedberg et al., 2009). Notably, pubMLST provides data on the penicillin susceptibility of 72 strains with the *penA52* allele. Of these, 19 strains (26.4%) are resistant to penicillin, with penicillin concentrations ranging from 0.064 mg/L to 0.640 mg/L (<https://pubmlst.org/>).

The meningococcal population structure is diverse and dynamic, with distinct strains grouped into various clonal complexes, some of which are associated with IMD (Caugant and Maiden, 2009). In Lithuania, genomic diversity analysis revealed 21 different circulating STs, with ST34 being the most prevalent (76.19%). Three isolates (ID 120132, 120136, and 120140) displayed unique combinations of housekeeping genes and were identified as novel STs (ST16969, ST16901, and ST16959). Importantly, these isolates with newly identified STs were obtained from adult individuals (ages 40–47 years). It is

noteworthy that IMD primarily affects infants in most countries (European Centre for Disease Prevention and Control, 2023).

Using the cgMLST scheme, MST revealed no significant geographic relationships. The ST-32 complex was most prevalent in Lithuania (58.1%) but less so in other European countries (7.9%). Lithuanian isolates in the ST-32 complex formed a distinct clade with isolates from the United Kingdom, Netherlands, Germany, France, Belarus, and Poland. Notably, the most prevalent clonal complex in Lithuania, CC32, is associated with hyperinvasive strains and global outbreaks. *N. meningitidis* CC32 caused outbreaks in the United Kingdom during the 1980s (Mikucki et al., 2022) and in the United States from 2013 to 2017 (Soeters et al., 2019). This study provides valuable insights into the epidemiological characteristics of IMD in Lithuania. To the best of our knowledge, this is the first study to investigate the genotypic relationship between Lithuanian isolates and strains isolated in Europe with the aim of determining the relatedness of Lithuanian isolates based on core-genome analysis.

Over the last decade, WGS has emerged as a leading technique in clinical microbiology (Purushothaman et al., 2022). The genomes of 10 distinct *N. meningitidis* strains were analyzed using the advancements offered by WGS to gain a better understanding of the biology of *N. meningitidis* and the epidemiology of IMD in Lithuania. Initially, we observed that the analyzed strains harbored a genome of approximately 2.2 million bases and a GC content of 51.7%, as previously described elsewhere (Caugant and Brynildsrud, 2019). KEGG analysis revealed highly similar pathway modules among the strains, with only minor differences observed. We found that two *N. meningitidis* strains displayed incomplete pathways for Shikimate and Siroheme biosynthesis, potentially affecting their metabolic capabilities and nutritional requirements. Additionally, only three strains possessed a complete pathway for dTDP-L-rhamnose biosynthesis. dTDP-L-rhamnose is crucial for building cell surface structures essential for bacterial interactions with the environment, including the host immune system (van der Beek et al., 2019). Further investigation of the specific genetic differences associated with this pathway in strains with complete and incomplete pathways could provide insights into the molecular basis of these variations and their potential impact on the biology, adaptation, and pathogenicity of *N. meningitidis*.

The initial bacterium-host interaction relies on adhesion, where specific bacterial surface molecules (adhesins) bind to host cell receptors. Various surface structures like pilus, opacity-associated proteins (Opa and Opc), autotransporter adhesins (App, MspA/AusI, NadA, and NhhA), two-partner secretion systems (HrpA and HrpB) play key roles. Additionally, other surface structures like polysaccharide capsules, lipo-oligosaccharides, and porins also contribute to adhesion (Hung and Christodoulides, 2013). Our results revealed combinations of adhesion domains and possibly adhesion-related domains in *N. meningitidis* isolates from Lithuania.

Previous studies have indicated that approximately 17% of *N. meningitidis* strains possess a Gonococcal Genetic Island (GGI).

However, some strains exhibit multiple insertions and deletions within the GGI, whereas others harbor intact T4SS genes, potentially leading to the production of functional secretion systems (Woodhams et al., 2012). During the domain analysis, we identified a strain with a unique combination of domains that was not found in the other strains examined. The domains identified in strain ID 120139 (TraL, TraE, TraK, TraC, TraU, type-F conjugative transfer system pilin assembly protein, F plasmid transfer operon protein, TraG-like protein, and N-terminal region) were encoded by GGI. Although it has been suggested that GGI is absent in serogroups A, B, and Y (Woodhams et al., 2012), our study found specific GGI sequences in the *N. meningitidis* serogroup Y.

The analyzed isolates possessed the essential virulence factors required for effective colonization and pathogenicity, affirming their disease-causing potential. The outer membrane vesicles, NhhA, and the capsules are among the most widely recognized and extensively studied virulence factors of *N. meningitidis* (Joshi and Saroj, 2023). According to the VFDB, the analyzed strains possessed virulence factors not commonly found in *N. meningitidis*, such as types of endotoxins (LOS) found in the genus *Haemophilus* and a capsule-associated virulence factor for antiphagocytosis found in the genus *Vibrio*. In this study, 82 genes linked to virulence were identified, most of which were associated with adherence and iron uptake, which is consistent with the well-established dependence of *N. meningitidis* growth on iron availability.

In addition to virulence factors, the analyzed isolates contained various antibiotic-resistance genes, providing resistance to multiple classes of antibiotics. *penA* gene mosaic alleles, characterized by four or five amino acid substitutions, have been shown to induce intermediate susceptibility to penicillins (Taha et al., 2007). Three isolates in our study harbored alterations in *penA*, encoding penicillin-binding protein 2, carrying E464A, E469K, N472E, F504L, A510V, I515V, H541N, A549T and I566V substitutions. These amino acid sites have been previously linked to decreased susceptibility to beta-lactam antibiotics, such as penicillin and cephalosporins (Hao et al., 2011). The isolate with the *penA*52 allele, for which the penicillin MIC was 0.125 µg/mL, had an asparagine insertion T573_A574insN in its *penA* gene. The significance of the Asn insertion in PBP2 is unclear (Du Plessis et al., 2008). To investigate the association between the asparagine insertion and penicillin susceptibility, we analyzed *penA* gene sequences from 209 *N. meningitidis* isolates with penicillin MICs above 0.25 µg/mL and 137 isolates with penicillin MICs equal to or below 0.016 µg/mL, sourced from the pubMLST database. Our findings indicate that 70% of the penicillin-resistant isolates contained an additional asparagine (AAT) codon in their *penA* genes. In contrast, none of the penicillin-susceptible isolates possessed this insertion (data not shown).

Another mechanism leading to penicillin resistance is the presence of *bla* genes that encode beta-lactamases (Tsang et al., 2019). The *bla*ROB-1 gene was not present in any of the analyzed isolates. Studies have shown that resistance to ciprofloxacin can be induced by

mutations in *parC* or the quinolone resistance-determining region of *gyrA* (Chen et al., 2020), while resistance to chemoprophylactic agents, such as rifampicin, due to mutations in *rpoB* has also been documented (Taha et al., 2010). No alleles were associated with phenotypic resistance to fluoroquinolones (*gyrA*, NEIS1320, NEIS1525, and NEIS1600) or rifampicin (NEIS0123 and *rpoB*). In the current study, we identified *N. meningitidis* strains with novel *penA*, NEIS0123, NEIS1320, NEIS1525, NEIS1600, and NEIS1753 locus variants. However, further exploration of antibacterial resistance using phenotypic and transcriptomic analyses is necessary to obtain a comprehensive understanding of how newly detected gene alleles correlate with antimicrobial sensitivity.

While *N. meningitidis* remains susceptible to antibiotics employed for the treatment and prevention of IMD, concerns persist regarding reduced antibiotic susceptibility (Rostamian et al., 2022). In our study, none of the analyzed isolates exhibited phenotypic resistance to penicillin, cefotaxime, rifampicin, or ciprofloxacin. However, the identification of several strains with genes associated with antimicrobial resistance raises the concern that resistance may emerge in the future, potentially altering the epidemiology of IMD in Lithuania.

In summary, this study evaluates IMD trends in Lithuania from 2009 to 2021. However, it has limitations. Firstly, the relatively small number of available genomes for WGS analysis may have limited the detection of unique genetic characteristics specific to strains circulating in Lithuania. Secondly, the data collected from a limited number of sources did not reflect the prevalence of *N. meningitidis* among carriers. Another limitation of this study relates to our genome assemblies being based on short-read sequencing only. This may introduce the possibility that some genes were not detected due to the incompleteness of the genome. However, our draft genomes exhibited high quality indicators (low contig number, expected genome size, and high coverage), which gives us confidence in our reported results. In future studies, PCR targeting the regions of interest or long-read sequencing technologies such as Nanopore sequencing, capable of generating ultra-long (>4Mb) reads, could be employed to enhance the reconstruction and characterization of genes in *N. meningitidis* isolates. Consequently, it is challenging to determine the distinct characteristics of MenB CC32 genomes in Lithuania, as observed through WGS, which distinguishes them from MenB CC32 and nonvirulent strains found in other regions. This limitation hinders our ability to reliably explain variations in virulence and pathogenicity. Finally, the identified genetic virulence, pathogenicity, and antibiotic resistance determinants have not been experimentally confirmed.

To the best of our knowledge, this is the first study to explore the genetic characteristics of *N. meningitidis* isolates from Lithuania and to investigate the genotypic relationship of Lithuanian isolates with strains isolated in Europe, with the aim of determining the relatedness of Lithuanian isolates based on core-genome analysis. Finally, this study enriches pubMLST database with WGS data from Lithuanian isolates. While Lithuania has not experienced any outbreaks thus far, it is essential to prioritize the molecular epidemiological surveillance of *N. meningitidis* as hypervirulent strains continue to emerge.

Data availability statement

The datasets generated and analyzed in this study have been deposited in the Public Databases for Molecular Typing and Microbial Genome Diversity (PubMLST). The genome sequences are accessible at the following link: https://pubmlst.org/bigdb?db=pubmlst_neisseria_isolates&page=query&genomes=1, with the accession numbers as follows: 119504, 120132, 120133, 120134, 120135, 120136, 120137, 120138, 120139, 120140.

Ethics statement

This study uses strains obtained from the National Public Health Surveillance Laboratory. The Lithuanian Bioethics Committee did not require the study to be reviewed or approved by an ethics committee because it did not involve human biological samples or patient-related information. This retrospective study exclusively used pure cultures of microorganisms, with each isolate sample encrypted and identified by a unique code to ensure anonymity. Under Lithuanian law, the use of microbial cultures in this study does not fall under the scope of biomedical research. It is important to note that the isolates used were collected as part of national-level pathogen epidemiological surveillance, conducted in accordance with Order No. V-1194 issued by the Ministry of Health of the Republic of Lithuania on December 18, 2013.

Author contributions

AS: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. MK: Formal analysis, Writing – review & editing. AK: Investigation, Writing – review & editing. GV: Writing – review & editing. PS: Data curation, Formal analysis, Writing – review & editing. JB: Writing – review & editing. KT: Investigation, Writing – review & editing. AP: Writing – review & editing. NK: Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1432197/full#supplementary-material>

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Acinetobacter baumannii infection in critically ill patients with COVID-19 from Tehran, Iran: the prevalence, antimicrobial resistance patterns and molecular characteristics of isolates

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Background: The COVID-19 pandemic has led to the excessive use of antimicrobials in critically ill patients. Infections caused by *Acinetobacter baumannii* have increased significantly both regionally and globally during the COVID-19 pandemic, posing dramatic challenges for intensive care unit (ICU) patients. This study aimed to determine the prevalence, antimicrobial resistance patterns, presence of selected antimicrobial resistance genes, and genetic diversity of *A. baumannii* isolates obtained from COVID-19 cases admitted to the ICU at the University Hospital in Iran.

Materials and methods: This was a cross-sectional and single-center study comprising patients with *A. baumannii* infections admitted to the ICU with COVID-19 between April and November 2021. The demographic and clinical data of the patients were collected. Antimicrobial susceptibility testing was conducted based on Clinical Laboratory Standards Institute guidelines. This study used PCR and multiplex PCR to investigate antibiotic resistance genes (ARGs) and global clones (GC), respectively. Genetic diversity was investigated by repetitive element sequence-based PCR (REP-PCR).

Results: The prevalence of *A. baumannii* coinfection in COVID-19 cases was 8.1% (43/528). More than 90% (39/43) of *A. baumannii* isolates were resistant to cefepime, ampicillin-sulbactam, gentamicin, trimethoprim-sulfamethoxazole and amikacin. Furthermore, 44.2% (19/43) of isolates were resistant to colistin. There were 91% (39/43) isolates that were extensively drug-resistant (XDR). The most prevalence carbapenem resistance encoding genes were bla_{OXA-23} 65.1% (29/43) and bla_{NDM} 41.8% (18/43). The most common aminoglycoside resistance genes were aac(6')-Ib 65.1% (28/43) and ant(2)-Ia 46.5% (20/43). Isolates from

the prominent Global clone GCII comprised 83.7% (36/43) of total isolates. Genetic fingerprinting using REP-PCR revealed that 39 typeable *A. baumannii* isolates were categorized into 12 distinct genotypes, of which 72% (28/39) of isolates belonged to one genotype.

Conclusion: The high prevalence of XDR *A. baumannii* such as carbapenem and colistin-resistant strains, poses a significant concern for the treatment of COVID-19 patients, heightening the risk of therapeutic failure. The data demonstrate the dissemination of a single *A. baumannii* clone carrying multiple ARGs within our hospital. Regarding the limited therapeutic options, it is crucial to implement effective prevention and containment policies to curb the spread of these strains.

KEYWORDS

Acinetobacter baumannii, SARS-CoV-2, co-infection, antibiotic resistance, resistance genes, global clones, ICU, REP-PCR

Introduction

Respiratory infections caused by acute coronavirus syndrome 2 (SARS-CoV-2) were first reported in December 2019 in Wuhan, China, and subsequently rapidly spread globally (Sharma et al., 2020). According to the World Health Organization (WHO) report dated December 30, 2023, the virus has been identified in more than 773 million individuals across 220 countries and has unfortunately caused the death of more than 7 million people (Organization WH, 2023). The clinical manifestations of coronavirus infection vary from asymptomatic infection to severe viral pneumonia, requiring treatment in an intensive care unit (ICU) or mechanical ventilation (Zeng et al., 2021). Hospitalization of COVID-19 patients, especially in the ICU, creates ideal conditions for acquiring healthcare-associated infections (HAIs) or secondary infections, particularly with multidrug-resistant (MDR) Gram-negative pathogens such as *Acinetobacter baumannii* (Ceparano et al., 2022). During the COVID-19 pandemic, there has been a significant increase in the prevalence of *A. baumannii* infections both regionally and globally (Boral et al., 2022; Mobarak-Qamsari et al., 2023; Petazzoni et al., 2023). This organism exhibits high rates of resistance to multiple antibiotics, including carbapenems, aminoglycosides, fluoroquinolones, and polymyxins, due to both rapidly acquired resistance genes and intrinsic resistance (Kyriakidis et al., 2021). In 2017, Carbapenem-resistant *A. baumannii* (CRAB) was listed at the top of WHO priorities for the development of new antibiotics (Tacconelli et al., 2018). Clonal spread of CRAB has been reported worldwide, with global clones 1 and 2 (GC1 and GC2) frequently associated with the increased resistance of this microorganism (Zarrilli et al., 2012). Infections caused by extensively drug-resistant (XDR) or MDR bacteria lead to higher ICU mortality and morbidity rates, along with increased

healthcare costs, while available therapeutic options are limited (Barbato et al., 2019). The treatment of patients with COVID-19 co-infected with antibiotic-resistant bacteria circulating frequently in hospitals is extremely challenging. Therefore, in order to combat antibiotic resistance and implement prevention control practices, the detection of antibiotic-resistant bacteria is essential (Lucien et al., 2021). The aim of the current study was to evaluate the prevalence, antibiotic resistance profiles and genetic relatedness of *A. baumannii* isolates obtained from patients with COVID-19 infection admitted to the ICU in a tertiary care hospital in Tehran, Iran.

Methods

Setting and sampling

This was a cross-sectional and single-center study, including patients admitted to Shariati Hospital, a tertiary referral hospital in Tehran, Iran, between April and November 2021. Patients were included in the current study based on the following criteria (Sharma et al., 2020): laboratory-confirmed SARS-CoV-2 infection using an RT-PCR test on a nasopharyngeal swab (Organization WH, 2023); unilateral or bilateral interstitial infiltrates confirmed by chest X-ray (Zeng et al., 2021); the presence of acute hypoxemic respiratory failure requiring mechanical ventilation. Infections were considered nosocomial if the positive culture for *A. baumannii* was obtained more than 48 hours after hospital admission. To distinguish true co-infection from colonization, a combination of clinical, microbiological, and diagnostic criteria was employed. Only patients with positive cultures from both clinically relevant sites (e.g., blood, sputum,

bronchoalveolar lavage (BAL), urine, wound) accompanied by signs and symptoms of infection, such as fever, respiratory distress, or sepsis, were considered to have co-infections. In contrast, patients with positive cultures from non-infectious sites (e.g., throat or rectal swabs) who did not exhibit clinical signs of infection were classified as colonized. Patient data were obtained from hospital computerized databases. The following information was collected: clinical, demographic, and laboratory findings; underlying conditions; microbiological data; duration of ICU stay.

Definitions

Pulmonary infections were classified into two categories: (a) co-infections, referring to cases where patients with confirmed COVID-19 simultaneously harbored other pathogens within the first 48 hours, and (b) secondary infections, characterized by the emergence of new pathogenic infections occurring beyond 48 hours following hospital admission (Lansbury et al., 2020). A bloodstream infection is identified by the presence of a likely pathogen in at least one positive blood culture. For organisms common to skin flora, multiple positive cultures (e.g., two or more) are often needed to confirm infection and rule out contamination (Timsit et al., 2020). Septic shock was defined according to the 2016 Third International Consensus Definitions for Sepsis and Septic Shock (Singer et al., 2016). Urinary tract infection was diagnosed based on both clinical and microbiological evidences (Van Laethem et al., 2021).

Isolate identification

One isolate per patient was included in this study. All bacterial isolates were identified using standard laboratory methods, including Gram-staining, oxidase and catalase tests, oxidative/fermentative tests, motility, and growth ability at 42°C (Vasconcellos et al., 2017). Species identification was confirmed by amplification of *gltA* gene (encoding species citrate synthase), as described previously (Wong et al., 2014). The *A. baumannii* isolates were stored at -70°C in trypticase soy broth with 20% glycerol until used for the study.

Antibiotic susceptibility testing

The antimicrobial susceptibility was determined using the Kirby Bauer (disc agar diffusion) method based on Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein MP et al., 2022). For disk diffusion test, eleven antimicrobial disks (MAST, United Kingdom) were used including: amikacin (30 µg), gentamicin (10 µg), cefepime (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (10 µg), trimethoprim-sulfamethoxazole (30 µg), ampicillin-sulbactam (30 µg), meropenem (10 µg) and imipenem (10 µg). The broth microdilution method was implemented to determine

the minimum inhibitory concentration (MIC) of colistin according to CLSI guidelines (Weinstein MP et al., 2022). Additionally, the broth disk elution method was used to confirm the resistance to colistin according to CLSI guideline *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The investigated isolates which were resistant to three or more different classes of antibiotics except for carbapenems, were considered as MDR phenotype and MDR isolates which were resistant to meropenem, were considered as XDR phenotype (Kyriakidis et al., 2021).

DNA extraction

To obtain genomic DNA, the boiling method was implemented as previously described (Chen et al., 2020). DNA purity and quantification was assessed using NanoDrop device (Thermo Scientific, Wilmington, DE, USA). The supernatant was stored at -20°C as the DNA template to be used in PCR reactions.

Detection of antibiotic resistance genes

The presence of class D carbapenemases genes (*bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like}) was investigated by multiplex-PCR method as described previously (Woodford et al., 2006). Furthermore, isolates harbouring the class B carbapenemases or metallo-beta-lactamases (MBL) genes (*bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM-1}), class A carbapenemase (*bla*_{KPC}, *bla*_{TEM}), aminoglycoside resistance genes (*aac*(6')-Ib, *aac*(3)-Ia, *ant*(2'')-Ia and *aph*(3')-Ia) and colistin resistance determinant (*mcr*-1) were detected by single PCR as described previously (Giakkoupi et al., 2003; Robicsek et al., 2006; Nguyen et al., 2010; Chen and Huang, 2013; Bina et al., 2015; Ghotaslou et al., 2017; Chen et al., 2020; Abdollahi et al., 2021; Pourajam et al., 2022). Positive and negative controls were used for each of the investigated ARGs during PCR. The primers and the PCR conditions used for the amplification are listed in Table 1.

Identification of global clones

Alleles of *ompA*, *csuE* and *OXA*_{51-like} genes were amplified using multiplex-PCR method in order to identify group 1 (GC2) or group 2 (GC1) as previously described (Turton et al., 2007). The primers sequences are provided in Table 2. Each PCR reaction mixture contained: 12.5 µL PCR Master Mix (Ampliqon, Denmark), which contains Taq DNA polymerase, PCR Buffer, and dNTPs, 1 µL of 20 pM of each primer (Metabion, Germany) and 100 ng of genomic DNA. Amplification reaction was performed by thermal cycler with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45s, annealing at 57°C for 45s, and extension at 72°C for 1 min, afterwards final extension at 72°C for 5 min. After visualization of products by gel electrophoresis, if all three genes (Table 2) associated with group I

TABLE 1 The primers and the conditions used for the amplification of genes encoding antibiotic resistance.

Gene	Primer sequence (5' to 3')	Product size (bp)	PCR programme*	Reference
<i>aac(6')-Ib</i>	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	94°C, 60s; 61°C, 40s; 72°C, 30s: 30 cycles	(Vasconcellos et al., 2017)
<i>aac(3)-Ia</i>	GCAGTCGCCCCATAAACAAA CACTTCTTCCCGTATGCCCAACTT	464	94°C, 60s; 61°C, 40s; 72°C, 30s: 30 cycles	(Wong et al., 2014)
<i>ant(2'')-Ia</i>	ACGCCGTGGGTCGATGTTTGATGT CTTTCCGCCCCGAGTGAGGTG	572	94°C, 60s; 67°C, 40s; 72°C, 35s: 30 cycles	
<i>aph(3') Ia</i>	CGAGCATCAAATGAAACTGC GCGTTGCCAATGATGTTACAG	624	94°C, 60s; 57°C, 40s; 72°C, 30s: 30 cycles	
<i>TEM</i>	TGCGGTATTATCCCGTGTTG TCGTCGTTTGGTATGGCTTC	297	94°C, 60s; 55°C, 40s; 72°C, 30s: 30 cycles	(Weinstein MP et al., 2022)
<i>VIM-1</i>	AGTGGTGAGTATCCGACAG ATGAAAGTGCGTGGAGAC	261	94°C, 60s; 56°C, 40s; 72°C, 30s: 30 cycles	(Chen et al., 2020)
<i>KPC</i>	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798	94°C, 60s; 60°C, 40s; 72°C, 40: 30 cycles	(Woodford et al., 2006)
<i>IMP</i>	CATGGTTTGGTGGTTCTTGT ATAATTGGCGGACTTTGGC	448	94°C, 60s; 56°C, 40s; 72°C, 30s: 30 cycles	(Abdollahi et al., 2021)
<i>NDM</i>	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	94°C, 60s; 62°C, 40s; 72°C, 35s: 30 cycles	(Bina et al., 2015)
<i>mcr-1</i>	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	94°C, 60s; 56°C, 40s; 72°C, 30s: 30 cycles	(Chen and Huang, 2013)
<i>OXA₂₃-like</i>	GATCGGATTGGAGAACCAGA ATTCTGACCGCATTTCCAT	501	94°C, 60s; 57°C, 45s; 72°C, 60: 30 cycles	(Timsit et al., 2020)
<i>OXA₂₄-like</i>	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	246		
<i>OXA₅₈-lik</i>	AAGTATTGGGGCTTGCTG CCCTCTGCGCTCTACATAC	599		

*Before the first cycle of gene amplification, the sample was subjected to denaturation at 94°C for a duration of 5 minutes. Following the final cycle, the sample underwent an extension phase at 72°C for 5 minutes.

TABLE 2 Primers used for identification of Global Clones.

Gene	Primer sequence (5' to 3')	Product size(bp)	Tm (°C)	Ref
<i>Group1ompA Forward</i>	GATGGCGTAAATCGTGGTA	355	55	(Chen and Huang, 2013)
<i>Group 1ompA Reverse</i>	CAACTTTAGCGATTTCTGG	355	53	
<i>Group 1csuE Forward</i>	CTTTAGCAAACATGACCTACC	702	57	
<i>Group 1csuE Reverse</i>	TACACCCGGGTTAATCGT	702	54	
<i>Group1OXA66 Forward</i>	GCGCTTCAAAAATCTGATGTA	559	54	
<i>Group1OXA66 Reverse</i>	GCGTATATTTTGTTTCCATTC	559	54	
<i>Group 2ompA Forward</i>	GACCTTTCTTATCACACGA	343	54	
<i>Group 2ompA Reverse</i>	CAACTTTAGCGATTTCTGG	343	57	
<i>Group 2csuE Forward</i>	GGCGAACATGACCTATTT	580	52	
<i>Group 2csuE Reverse</i>	CTTCATGGCTCGTTGGTT	580	54	
<i>Group2OXA69 Forward</i>	CATCAAGGTCAAACCTCAA	162	49	
<i>Group2OXA69 Reverse</i>	TAGCCTTTTTTCCCCATC	162	52	

are detected as positive, the isolates are classified under group 1. Similarly, if all three genes associated with group II are positive, the isolates are classified under group 2. In cases when *ompA* from group II, along with *csuE* and *OXA66* from group I, are positive, the isolates are classified under group 3 of the global clone.

Repetitive Extragenic Palindromic Element PCR (Rep-PCR) Genotyping

A. baumannii isolates were subjected to molecular typing by REP-PCR using the primer pairs REP1R-I (IIIICGICGICATCIGGC) and REP 2-I (ICGICTTATCIGGCCTAC) which were implemented to amplify putative REP like elements in the bacterial chromosomes (Chen and Huang, 2013). DNA amplification was performed in a final volume of 25 µl containing 12.5 µl of 2X Multi-Star PCR Master Mix (Bio-FACT, South Korea), 1 µl of each primer, 8.5 µl of distilled water, and 2 µl of the template DNA. Amplification reaction was performed by thermal cycler with an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min, afterwards final extension at 72°C for 16 min. After visualization of products by gel electrophoresis, REP-PCR patterns were analyzed Using http://insilico.ehu.es/dice_upgma/ and isolates that revealed similarity cut-off $\geq 80\%$ of their banding patterns were considered as the same types and isolates with similarity cut-off $< 80\%$ were taken as different types.

Statistical analysis

All data regarding the results of microbial tests, clinical findings and demographic characteristics, were added to the statistical package IBM SPSS Version 26 (Armonk, NY, USA) and analysis was performed using descriptive statistical tests.

Results

Prevalence of *A. baumannii* infections in Patients with COVID-19

Between April and November 2021, a total of 3,868 patients were admitted to intensive care units (ICUs), of whom 528 were diagnosed with PCR-confirmed COVID-19. Among these, 8.1% (43/528) were infected with *A. baumannii* and were included in this study. All isolates were confirmed to be *A. baumannii* by amplifying the *gltA* gene. Of 43 *A. baumannii* isolates, 81.4% (35/43) were recovered from respiratory samples, 14% (6/43) from blood, and 4.6% (2/43) from urine samples. The majority of isolates were recovered from males, comprising 62.8% (27/43). The age range was 18 to 87 years, with a mean age of 66.7 years. The most prevalent comorbidity in patients was hypertension, affecting 48.8% (21/43), followed by chronic heart disease in 39.5% (17/43), and diabetes in 30.2% (13/43). Ultimately, at the end of a median length of stay of 26.2 days, 88.4% (38/43) patients had died (Table 3). Meropenem

TABLE 3 Demographic Characteristics and laboratory findings of Hospitalized COVID-19 Patients (n=43) with *A. baumannii* coinfection.

Parameters		(Mean $\pm$ SD)
Age		66.7 $\pm$ 11.1
Duration of Hospital Stay		26.2 $\pm$ 27/1
Laboratory findings on admission	CRP (mg/L)	110 $\pm$ 42.6
	ESR	89 $\pm$ 36.3
	WBC	11800 $\pm$ 19591
	Platelet count	164 $\pm$ 102
		n (%)
Gender	Male	27 (62.8)
	Female	16 (37.2)
Outcome	Expired	38 (88.4)
	Discharged	5 (11.6)
Sample source	Respiratory tract samples	35 (81.4)
	Blood	6 (14)
	Urine	2 (4.6)
Comorbidities	Diabetes	13 (30.23)
	Hypertension	21 (48/84)
	Chronic heart disease	17 (39.53)
	Chronic respiratory disease	1 (2.33)
	Immunodeficiency	7 (16.28)
	Cancer	4 (9.3)
	addiction	3 (6.98)
	Chronic kidney disease	5 (11.63)

was administered to all patients in the current study. Additionally, 91% (39/43) of the patients received a combination therapy of meropenem and colistin.

Antimicrobial susceptibility tests

All 43 *A. baumannii* isolates were tested against a panel of 11 antibiotic discs and colistin as recommended by the CLSI-2021. The frequency of resistance to most tested antibiotics was high. Among the 43 *A. baumannii* strains isolated from COVID-19 patients, the highest resistance rate was observed against cefotaxime 100% (43/43), followed by 97.7% (42/43) resistance against each imipenem, meropenem, piperacillin-tazobactam and ciprofloxacin. The resistance rate against cefepime and ampicillin-sulbactam was 95.3% (41/43), 93% (40/43) against gentamicin, 90.7% (39/43) against each trimethoprim-sulfamethoxazole and amikacin and 88.4% (38/43) against ceftazidime. The most active antimicrobial agent against *A. baumannii* strains from COVID-19 patients was

colistin with 55.8% (24/43) sensitivity. Most of the isolates, 91% (39/43), were XDR (resistant to +3 antibiotic classes), and 9% (4/43) were MDR phenotypes. Ten distinct patterns of antibiotic resistance were identified among 43 *A. baumannii* strains from COVID-19 patients in which the most prevalent patterns were resistant to all tested antibiotics except colistin 44.2% (19/43) and resistant to all tested antibiotics 34.8% (15/43), respectively (Table 4).

Detection of ARGs

The rate of genes encoding class D carbapenemases which were detected using Multiplex PCR was *OXA*_{23-like} 67.4% (29/43), *OXA*_{24-like} 30.2% (13/43) and *OXA*_{58-like} 7% (3/43). Amongst the MBLs; *bla*_{NDM} was detected in 41.8%, (18/43), *bla*_{IMP} 2.3% (1/43) and *bla*_{VIM-1} 9.3% (4/43) isolates. Class A β-lactamase *bla*_{KPC} was detected in 9.3% (4/43) isolates and 48/8% (21/43) of isolates harbored *bla*_{TEM} gene. The following percentages of selected genes encoding aminoglycoside-modifying enzymes (AMEs) [*aac*

(6′)-*Ib* 65.1% (28/43), *aac*(3′)-*Ia*, 4.7% (2/43), *ant*(2′)-*Ia* 46.5% (20/43), *aph*(3′)-*Ia* 16.3% (7/43)] were observed among analyzed strains. PCR amplification of *mcr-1* primers did not bring about a product for colistin resistance encoding gene. Our study shown the presence of at least one AME and β-lactamase encoding gene in 88.37% (38/43) and 67.4% (29/43) of isolates, respectively (Table 4).

Global clones lineages

Multiplex PCR for the identification of GCs indicated 83.7% (36/43), 11.6% (5/43) and 4.7% (2/43) of *A. baumannii* isolates belonged to GC II, GC I and GC III, respectively (Figure 1).

REP-PCR genotyping

The clonal relatedness of 43 *A. baumannii* isolates was studied by REP-PCR, which amplified 5 to 9 bands with molecular weight

TABLE 4 The demographic, molecular characteristics and antimicrobial resistance profile of 43 *Acinetobacter baumannii* isolates obtained from patients with Covid-19 infection.

Isolate No.	Sample source	Gender	Outcome	Resistance pattern	Resistance gene	REP type
AB2	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>VIM</i> , <i>TEM</i>	G
AB3	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>OXA</i> _{23-Like}	Untypeable
AB5	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>aac6′Ib</i>	G
AB6	BAL	Male	Expired	IMI, MEM, SAM, TS, CTX	<i>aac3-Ia</i> , <i>OXA</i> _{23-Like}	D
AB7	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>NDM</i> , <i>VIM</i> , <i>KPC</i> , <i>TEM</i> , <i>OXA</i> _{23-Like}	F
AB8	Urine	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM, COL	<i>aac6′Ib</i> , <i>NDM</i> , <i>TEM</i> , <i>OXA</i> _{23-Like}	C
AB12	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CIP, GM, AK, SAM, COL, PTZ, CPM	<i>aph3′Ia</i> , <i>ant2′Ia</i> , <i>OXA</i> _{23-Like} , <i>OXA</i> _{58-Like}	Untypeable
AB19	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>OXA</i> _{23-Like}	G
AB24	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>aac6′Ib</i> , <i>NDM</i> , <i>VIM</i> , <i>KPC</i>	H
AB28	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>OXA</i> _{24-Like}	G
AB33	Blood	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>ant2′Ia</i> , <i>TEM</i> , <i>OXA</i> _{23-Like}	J
AB34	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>ant2′Ia</i> , <i>OXA</i> _{23-Like}	A
AB36	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6′Ib</i> , <i>ant2′Ia</i> , <i>aph3′Ia</i> , <i>NDM</i> , <i>OXA</i> _{23-Like}	G

(Continued)

TABLE 4 Continued

Isolate No.	Sample source	Gender	Outcome	Resistance pattern	Resistance gene	REP type
AB37	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6' Ib, aac3-Ia, aph3' Ia, NDM, OXA-23-Like</i>	G
AB39	Teracheal discharge	Male	Discharged	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>aac6' Ib, ant2' Ia, NDM, TEM, OXA-23-Like, OXA-58-Like</i>	G
AB42	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>OXA-24-Like</i>	G
AB47	Teracheal discharge	Female	Discharged	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6' Ib, ant2' Ia, OXA-23-Like</i>	B
AB48	blood	Male	Discharged	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6' Ib, ant2' Ia, OXA-23-Like, NDM</i>	G
AB50	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, SAM, PTZ, CPM	<i>aac6' Ib, ant2' Ia, OXA-24-Like</i>	G
AB51	Blood	Male	Expired	IMI, MEM, PTZ, CIP, CPM, GM, CTX, AK	<i>aac6' Ib, ant2' Ia, OXA-24-Like</i>	G
AB53	Teracheal discharge	Male	Expired	IMI, MEM, PTZ, CIP, CPM, GM, CTX, AK	<i>aac6' Ib, aac3-Ia</i>	G
AB57	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>VIM</i>	G
AB58	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>aac6' Ib, aph3' Ia, NDM, TEM</i>	G
AB59	Blood	Female	Expired	IMI, MEM, CTX, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>OXA-48, aac6' Ib</i>	G
AB60	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6' Ib, KPC, TEM, OXA-23-Like</i>	G
AB62	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>aac6' Ib, aph3' Ia, TEM, OXA-23-Like</i>	G
AB68	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>ant2' Ia, aph3' Ia, NDM, VIM, OXA-24-Like</i>	E
AB69	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>NDM, aac6' Ib,</i>	G
AB72	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aac6' Ib, TEM, OXA-24-Like</i>	I
AB75	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>TEM</i>	K
AB76	Blood	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>aph3' Ia, TEM, OXA-23-Like, OXA-24-Like</i>	G
AB78	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>ant2' Ia, IMP</i>	L
AB79	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>ant2' Ia, OXA-23-Like</i>	Untypeable
AB80	BAL	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>ant2' Ia, TEM, OXA-23-Like</i>	G
AB84	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	<i>TEM, OXA-24-Like</i>	G
AB85	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>ant2' Ia, TEM, OXA-23-Like</i>	G
AB86	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, PTZ, CPM	<i>ant2' Ia, TEM, OXA-23-Like</i>	G

(Continued)

TABLE 4 Continued

Isolate No.	Sample source	Gender	Outcome	Resistance pattern	Resistance gene	REP type
AB89	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	NDM, TEM, OXA-24-Like	G
AB96	Teracheal discharge	Female	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	ant2' Ia, TEM, OXA-24-Like	G
AB98	Teracheal discharge	Male	Discharged	IMI, MEM, CTX, CAZ, CIP, TS, SAM, COL, PTZ, CPM	aac6' Ib, TEM, OXA-23-Like	Untypeable
AB105	Teracheal discharge	Male	Expired	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	aac6' Ib, ant2' Ia, TEM, OXA-24-Like	G
AB109	Teracheal discharge	Male	Expired	PTZ, SAM, CIP, TS, CAZ, CTX	aac6' Ib, OXA-58-Like	G
AB118	Wound	Female	Discharged	IMI, MEM, CTX, CAZ, CIP, GM, AK, TS, SAM, COL, PTZ, CPM	OXA-24-Like	G

BAL, Broncho Alveolar Lavage; IMI, Imipenem; MEM, Meropenem; PTZ, Piperacillin-tazobactam; SAM, Ampicillin-sulbactam; CTX, Cefotaxime; CAZ, Ceftazidime; CIP, Ciprofloxacin; GM, Gentamicin; AK, Amikacin; TS, Trimethoprim-sulfamethoxazole; COL, Colistin; CPM, Cef.

ranging from 150 bp to 1.2 kb and were labelled A to L. Among 43 *A. baumannii* isolates, 39 isolates were typed by REP-PCR and no bands were observed in 4 isolates (Figure 1). By using a cut off value of $\geq 80\%$ as the threshold, 12 patterns among 39 tested isolates were observed, in which 11 patterns were detected once, while the remaining 1 pattern was repeatedly observed. Genotype G was the most prevalent, accounting for 72% (28/39) of the isolates. Of this genotype, 96% (27/28) exhibited resistance to carbapenems.

Discussion

Given the increased incidence of infections caused by MDR and XDR strains of *A. baumannii*, this pathogen has emerged as a significant threat to vulnerable ICU patients, especially those facing critical conditions, such as COVID-19 patients. In this cross-sectional study, the prevalence of *A. baumannii* co-infection in COVID-19 patients was 8.1%, which was similar with other reports from Iran

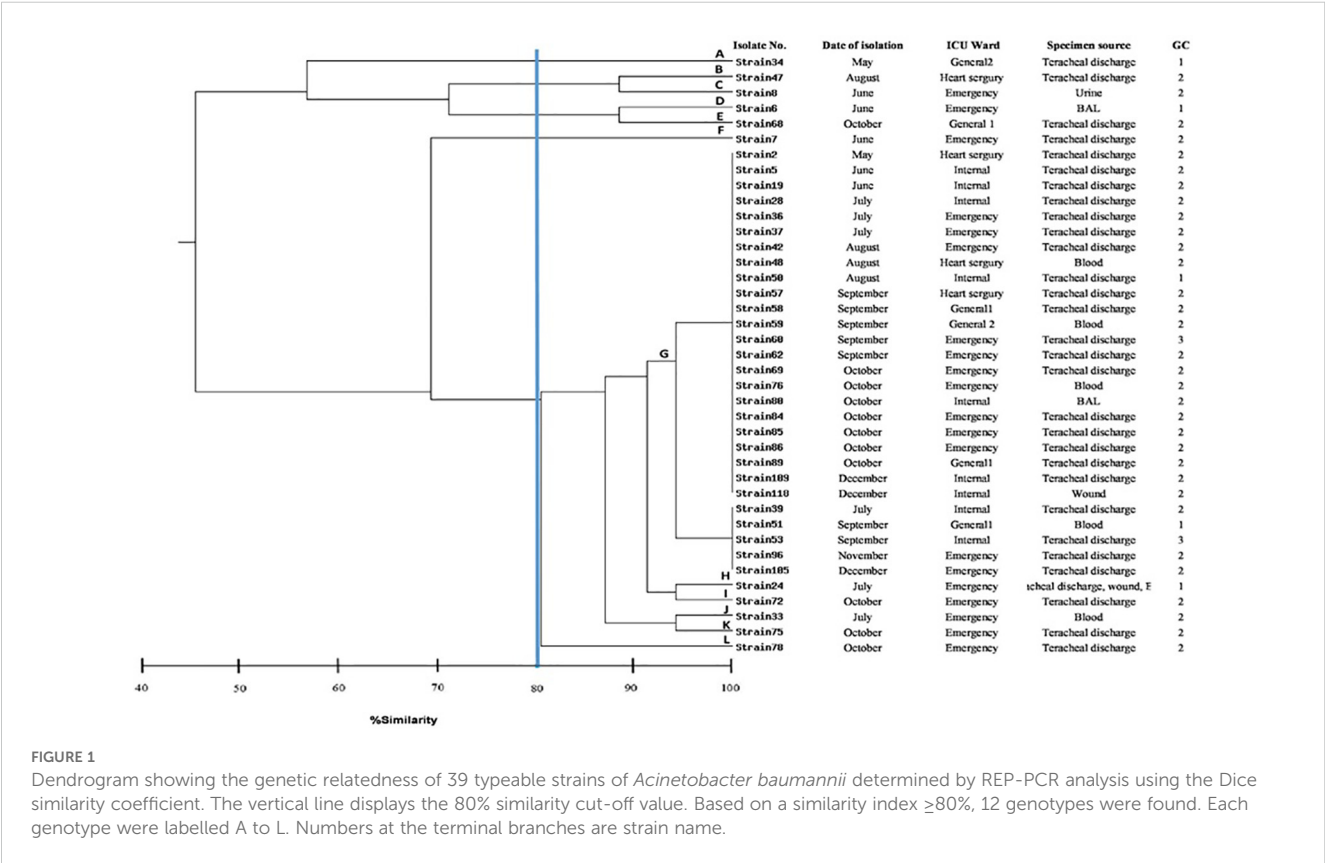


FIGURE 1 Dendrogram showing the genetic relatedness of 39 typeable strains of *Acinetobacter baumannii* determined by REP-PCR analysis using the Dice similarity coefficient. The vertical line displays the 80% similarity cut-off value. Based on a similarity index $\geq 80\%$, 12 genotypes were found. Each genotype were labelled A to L. Numbers at the terminal branches are strain name.

(7.44%) (Abdollahi et al., 2021), Jordan (8.1%) (Alsheikh et al., 2022) and India (8.9%) (Khurana et al., 2021); whereas, in other studies conducted in Italy (15.5%) (Cultrera et al., 2021), China (21.8%) (Sang et al., 2021), Iran (41% and 51%) (Jamnani et al., 2022; Pourajam et al., 2022) and United States (62%) (Perez et al., 2020), the incidence of co-infection was significantly higher than the rate of present Study. The observed variation may be attributed to the differences in healthcare systems and infection control practices (Organization WH, 2016), patient demographics and characteristics (Grasselli et al., 2020b), diagnostic methods and surveillance (Antibiotic resistance threats in the United States, 2019, 2019), regional epidemiology (Peleg et al., 2008) and study design and methodology (von Elm et al., 2007). Our study reveals that the number of males infected with *A. baumannii* in ICU was higher than the number of females infected with *A. baumannii*. This may be due to the differences in immunologic reaction because testosterone has immunosuppressive effect in males while estradiol has a pro-inflammatory effect in females (Hafiz et al., 2023). The mortality rates among COVID-19 patients varied widely across different countries, ranging from 16% to 100% (Grasselli et al., 2020a; Novović et al., 2023). Our study revealed a high mortality rate (88.4%) among COVID-19 patients infected with *A. baumannii*. A study conducted in Qom, Iran, Sharifipour et al. observed that among 17 COVID-19 patients with *A. baumannii* infections, 17 (100%) died. In another study in Serbia showed the mortality rate was 100% (Novović et al., 2023), while a study in South Korea had a lower mortality rate of 64.3% (Kim et al., 2023). Possible explanations for these differences in mortality rates might be attributed to variation in clinical supervision, the accessibility of therapeutics, healthcare organization and staff training (Malaeb et al., 2023). Additionally, comorbidities also play a crucial role in the mortality of COVID-19 patients. Hypertension, cardiovascular disease, and diabetes were the most common comorbidities observed in our study, consistent with similar findings elsewhere (Woodford et al., 2006; Abdollahi et al., 2021). In this study, the median length of stay in the ICU was higher (26.2 days) compared with other studies from Iran (15.8 days), South Korea (16.8 days) and Italy (12 days) (Grasselli et al., 2020a). The variation in the median length of stay might be related to several factors, including differences in healthcare systems, treatment protocols, patient demographics, and the prevalence and resistance patterns of *A. baumannii* (Vincent et al., 2014; Rosenthal et al., 2020). Regarding antibiotic resistance, our isolates were extremely resistant to the most evaluated antibiotics. Furthermore, out of the 43 isolates, 98% were CRAB, 91% were XDR and 9% were MDR. This high rate of antibiotic resistance in our hospital was possible as a result of the excessive or misuse of antibiotics, which creates the selection pressure for development of resistance. Alarming, 44.2% of our isolates were resistant to colistin, that is considered to be high with respect to previous studies (Boral et al., 2019; Ibrahim, 2019; Hafiz et al., 2023). In spite of high resistance rate to colistin, none of the isolates in present study harbored *mcr-1* gene. It seems that other colistin resistance mechanisms reported in *A. baumannii*, including mutation of the PmrAB system and loss of lipopolysaccharide (LPS) production might be involved in the resistance (Palmieri

et al., 2020; Zafer et al., 2023). In this study, we identified various ARGs, and the most were *OXA*_{23-like} (67.4%), *aac*(6')-Ib (65.1%), *bla*_{TEM} (48.8%), *ant*(2')-Ia (46.5%), *bla*_{NDM} (41.8%) and *OXA*_{24-like} (30.2%). In a previous study from the southwestern of Iran, Farajzadeh et al. observed that *OXA*_{23-like} (32.2%), *bla*_{VIM} (31.4%), *bla*_{IMP} (25.7%) were the most common carbapenemase genes among clinical isolates of *A. baumannii* (Farajzadeh Sheikh et al., 2020). In Egypt, Benmahmod et al. reported that *OXA*_{23-like} (94%), *bla*_{KPC} (56%) and *bla*_{NDM} (30%) were the most prevalent carbapenemase genes among *A. baumannii* strains isolated from clinical samples (Benmahmod et al., 2019). In Saudi Arabia, Alyamani et al. observed that *OXA*_{23-like} (91%) and *bla*_{TEM} (71%) were the most prevalent carbapenemase and class A β-lactamase encoding genes among *A. baumannii* isolates (Alyamani et al., 2015). In a Chinese study, 100%, 100%, 67.53% and 31.17% of the CRAB harbored *bla*_{VIM}, *OXA*_{23-like}, *bla*_{IMP} and *bla*_{NDM} genes, respectively (Zhu et al., 2022). In Algeria, the most prevalent AME genes in *A. baumannii* isolates were *aac*(3)-Ia (91.1%) and *aph*(3')-VI (50.7%) (Bakour et al., 2013). A study from China, Nie et al. reported that *ant*(3')-I (66.47%), *aac*(3)-I (45.09%), *aph*(3')-I (34.1%) and *aac*(6')-Ib (32.37%) were the most prevalent AME genes among *A. baumannii* isolates (Nie et al., 2014). As mentioned earlier, the prevalence of ARGs varied widely between different countries. These differences may be attributed to different patterns in use of antimicrobial agents, horizontal spread of resistance determinants, dissemination of specific clones harboring various types of ARGs and the number of studied isolates. In the current study, 8 (18.6%) isolates were negative for the seven carbapenemases tested, indicating that other mechanisms such as porin loss, overexpression of efflux pumps, AmpC enzymes might contribute to carbapenem resistance (may lead to resistance to carbapenems), which were not investigated in present study (Cai et al., 2019). Our data exhibited the coexistence of carbapenemases and aminoglycoside resistance genes among CRAB isolates, similar to previous reports from India (Karthikeyan et al., 2010) and Iran (Beig et al., 2023). These findings highlighted the difficulty in treating CRAB due to the presence of multiple ARGs. Infections with CRAB carrying multiple ARGs are usually associated with a high level of mortality and morbidity. As reported previously, the majority of *A. baumannii* isolates that are MDR and XDR belong to two international clones (GC1 and GC2) (Hamed et al., 2022). In agreement with other studies (Al-Sultan et al., 2015; Abdollahi et al., 2021), the current study demonstrated the predominance of GC2 isolates in our collection (83.7%). Molecular typing is a relevant tool for epidemiological purposes and examining the genetic structure of the organisms (Hallin et al., 2012). To further examine the genetic relatedness of *A. baumannii* isolates, the REP-PCR typing method was employed. In this work, 43 isolates were examined, leading to the identification of 12 distinct patterns among the 39 typeable isolates. Notably, a significant proportion (72%) of our isolates were grouped into a single genotype or clone, suggesting that these isolates were closely related and the spread of these isolates were associated with a clonal outbreak. It should be noted that we observed that isolates belonging to genotype G have the distinct ARGs, indicating that the horizontal transmission may occurred.

Generally, isolation of many resistant bacteria in hospitals such as CRAB can be driven by two epidemiological scenarios: the emergence and spread of a specific clone, or the persistence and coexistence of multiple clones (Al-Sultan et al., 2015). Our data are in concordance with the first scenario, because one clone is circulating in our hospital. To prevent the circulation of specific clones in hospitals, effective infection prevention and control are crucial. The hospital infection control committee (HICC) is present in the majority of Iranian hospitals, but HICC lacks an efficient program for infection prevention and control (Emaneini et al., 2018; Esfandiari et al., 2018). These committees exist generally on paper; in practice, they scarcely exist (Mamishi et al., 2014; Emaneini et al., 2018; Esfandiari et al., 2018). These issues are exacerbated during crises like the COVID-19 pandemic, potentially facilitating the horizontal transmission and spread of specific clones of multidrug-resistant organisms (Endale et al., 2023). It should be emphasized that the present study had several limitations. First, the major limitation of this study was its retrospective design. Second, owing to the small sample size and single-center design, the findings may not be generalizable to patient populations in different hospitals and countries. Third, utilization other typing approaches such as multi-locus sequence typing and pulsed-field gel electrophoresis for further genotypic characterization is recommended.

Conclusion

The high prevalence of MDR *A. baumannii* such as carbapenem and colistin-resistant strains, poses a significant concern for the treatment of COVID-19 patients, heightening the risk of therapeutic failure. The REP-PCR typing data demonstrate the dissemination of a single *A. baumannii* clone carrying multiple ARGs within our hospital. Regarding the limited therapeutic options, it is crucial to implement effective prevention and containment policies to curb the spread of these isolates.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/

institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because No clinical samples were drawn for this study, clinical samples collected as routine clinical care for these patients and remnant of samples were used. Therefore, informed consent was not sought, and informed consent waiver was approved by the ethics committee of Tehran University of Medical Sciences.

Author contributions

MG: Investigation, Methodology, Writing – original draft. FJ: Resources, Writing – review & editing. SA: Writing – review & editing. SH: Formal analysis, Software, Writing – review & editing. ME: Writing – review & editing. RB: Supervision, Writing – original draft.

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Conflict of interest

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

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Enhanced invasion and survival of antibiotic-resistant *Klebsiella pneumoniae* pathotypes in host cells and strain-specific replication in blood

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Background: *Klebsiella pneumoniae* is one of the most important opportunistic pathogens causing healthcare-associated and community-acquired infections worldwide. In recent years, the increase in antibiotic resistance and infections caused by hypervirulent *K. pneumoniae* poses great public health concerns. In this study, host-pathogen interactions of different *K. pneumoniae* strains of human and animal origins were analyzed in microbiological, cell-biological and immunological experiments.

Methods: *In vitro* infection experiments using representatives of different *K. pneumoniae* pathotypes and various epithelial and macrophage cell lines were executed analyzing adhesion, invasion and intracellular replication. Experimental conditions involved normoxia and hypoxia. Furthermore, survival and growth of further *K. pneumoniae* isolates expressing defined siderophores in blood (platelet concentrates, serum) was investigated. All experiments were done in triplicate and statistically significant differences were determined.

Results: Significant differences in adhesion and invasion capability, phagocytosis resistance and intracellular replication were measured between different *K. pneumoniae* pathotypes. Especially, ESBL-producing *K. pneumoniae* isolates demonstrated increased invasion in host cell lines and survival in macrophages. A strong cytotoxic effect on intestinal cells was observed for hypervirulent *K. pneumoniae*. The results from our investigations of the growth behavior of *K. pneumoniae* in platelets and serum showed that siderophores and/or an enlarged capsule are not essential factors for the proliferation of (hypervirulent) *K. pneumoniae* strains in blood components.

Conclusion: Our *in vitro* experiments revealed new insights into the host-pathogen interactions of *K. pneumoniae* strains representing different pathovars and clonal lineages in different infectious contexts and hosts. While

a clear limitation of our study is the limited strain set used for both infection and as potential host, the results are a further step for a better understanding of the pathogenicity of *K. pneumoniae* and its properties essential for different stages of colonization and infection. When developed further, these results may offer novel approaches for future therapeutics including novel “anti-virulence strategies”.

KEYWORDS

adhesion, invasion, replication, *Klebsiella pneumoniae*, serum resistance, platelet concentrate, cell morphology, normoxia

Introduction

Klebsiella pneumoniae is a Gram-negative bacterium that has emerged as a significant human pathogen. It poses a serious threat to public health by causing a wide range of infections in humans, including pneumonia, urinary tract infections, and bloodstream infections (Rahi et al., 2024). Originally recognized for its role in causing pneumonia more than 100 years ago, this microorganism has recently gained renewed attention due to its ability to adapt and evolve, resulting in enhanced virulence and evasion of host immune responses (Lam et al., 2018b; Martin and Bachman, 2018). Central to the pathogenicity of *K. pneumoniae* is its polysaccharide capsule, a complex outer layer that shields the bacterium from host defenses and contributes to its survival in diverse environments (Domenico et al., 1994; Sahly et al., 2000; Opoku-Temeng et al., 2019).

In the past 20 years, a continuous increase of *K. pneumoniae* with resistance to cephalosporines and carbapenems has been detected in healthcare settings worldwide. Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to a broad range of beta-lactam antibiotics, including penicillins and first- to third-generation cephalosporins (Fostervold et al., 2022; Hawkey et al., 2022). The prevalence of ESBL-producing *K. pneumoniae* strains from invasive infections varies considerably. For instance, rates of invasive *K. pneumoniae* with ESBL phenotypes ranged among European countries in 2021 from <5% to >80% with 34% on average and an overall increasing trend in recent years (Anonymous, 2023). Carbapenems are one of the few remaining treatment options for ESBL-producing and other drug-resistant *Klebsiella* infections. Thus, the emergence of carbapenem resistance is of major concern because the treatment options are limited only to a few remaining substances (Isler et al., 2022; Ding et al., 2023). Carbapenem resistance in *K. pneumoniae* is often mediated by the acquisition of carbapenemases of various types including KPC, OXA, and NDM (Argimón et al., 2021; Gorrie et al., 2022; Ding et al., 2023).

Hypervirulence in *K. pneumoniae* is a phenomenon characterized by strains that are able to cause severe infections in healthy individuals, in contrast to the usual susceptibility of immune-compromised patients to bacterial infections by classical

healthcare pathogens (Russo and Marr, 2019; Wyres et al., 2020; Russo et al., 2024). One defining feature of hypervirulent *K. pneumoniae* is hypermucoviscosity, attributed to an increased production of capsular polysaccharides. This hypermucoviscous phenotype is associated with specific virulence factors such as the presence and expression of the regulators of the mucoid phenotype *rmpA* and/or *rmpA2*, which finally enhances capsule production, providing protection against phagocytosis and contributing to the overall virulence of the bacterium (Russo and Marr, 2019; Wyres et al., 2020; Mendes et al., 2023; Russo et al., 2024).

The role of siderophores in the virulence of hypervirulent *K. pneumoniae* strains has attracted significant attention in recent studies (Russo et al., 2021; Kumar et al., 2024; Wahl et al., 2024). Siderophores are small molecules that help bacteria acquire iron, an essential nutrient for their survival and pathogenicity. *K. pneumoniae*, like many other pathogens, utilizes siderophores to scavenge iron from the host environment, thereby enhancing its ability to colonize the host and cause infection. These siderophores not only contribute to the bacterial iron acquisition but also play a significant role in modulating the host immune response, ultimately increasing the pathogenicity of the bacterium (Bachman et al., 2012; Holden et al., 2016; Russo et al., 2021; Mendes et al., 2023). Several types of siderophores have been identified including colibactin *clb*, yersiniabactin *ybt*, salmochelin *iro*, and aerobactin *iuc*, among others. Each siderophore type plays a distinct role in iron acquisition and may have different implications for the pathogenicity of *K. pneumoniae*. Kleborate, a tool widely used for the genomic characterization of *Klebsiella* isolates, assesses the virulence potential of *K. pneumoniae* strains based on the presence of specific siderophores and assigns a virulence score (Lam et al., 2021; Russo et al., 2024); whereas the tool Kaptive delineates the capsule and polysaccharide composition (Lam et al., 2022). Traditionally, hypervirulent *K. pneumoniae* were less resistant to antibiotics, making them susceptible to common treatments. However, the acquisition of ESBL or carbapenemase genes by these hypervirulent strains not only enhances their ability to cause severe disease but also limits therapeutic options tremendously (Wyres et al., 2020; Mendes et al., 2023; Merla et al., 2024; Wahl et al., 2024). Specific world regions are

primarily affected by this converging effect, in other regions and countries these strains occur sporadically (Liao et al., 2020; Hu et al., 2024; Merla et al., 2024; Wahl et al., 2024; Zhou et al., 2024).

Only a few studies investigated the association between increased antimicrobial resistance and virulence in *K. pneumoniae* (Mendes et al., 2023). Sahley et al. described that the acquisition of resistance-encoding plasmids is associated with increased fimbrial expression and increased adhesion to epithelial cells (Sahly et al., 2008). Another study investigated pathophysiological differences between antibiotic-susceptible, classical *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (hvKp) and demonstrated that hvKp resisted phagocyte-mediated clearance and replicated in mouse liver macrophages (Wanford et al., 2021). In the present study we follow previous observations and extend these analyses by providing new data about pathogen-host interactions of various *K. pneumoniae* strains of human and animal origin. Life-threatening *K. pneumoniae* infections include the capability of the pathogen to adhere to surfaces, to invade host cells and to translocate through cellular barriers. Therefore, we investigate adhesion, invasion and replication capacities to various human and animal cell lines as well as the phagocytosis resistance of cKp, ESBL-producing *K. pneumoniae* (ESBL-cKp) and hvKp isolates. The cell assays were carried out under normoxia and hypoxia conditions, the latter to better reflect the natural habitat of the bacterium. Furthermore, the resistance and growth in platelet concentrates and in human sera were investigated mimicking the infectious lifecycle of *K. pneumoniae*. The aim was to identify potential host associations of *K. pneumoniae* and to reveal novel findings about host adaptation of different pathotypes contributing to their pathogenicity and survival under challenging immunological conditions.

Materials and methods

Bacterial isolates and antibiotic susceptibility testing

We used the following isolates of *K. pneumoniae* for most of the functional assays: 17-0683 and 17-0684, representing antibiotic-susceptible classical *K. pneumoniae* (cKp); two ESBL-producing isolates (16-0382 and 16-0383; ESBL-cKp) and two hypervirulent isolates (17-0609 and 18-0005; hvKp). Isolates 17-0683 and 17-0684 were isolated from blood cultures and were susceptible to all antibiotics tested, except ampicillin. Ampicillin resistance is mediated by an SHV-type beta-lactamase that is characteristic of the *K. pneumoniae* species (Livermore, 1995). The ESBL-producing isolates 16-0382 and 16-0383 originated from an urine culture and a throat swab, respectively. Both isolates were resistant to third-generation cephalosporins and possessed the ESBL gene *bla*_{CTX-M-15}. The two selected hvKp isolates 17-0609 and 18-0005 belonged to the sequence types ST2398 and ST66, respectively (Klaper et al., 2021). Both isolates showed capsule type K2 and carried the virulence genes *ybt*, *iro*, *iuc*, *clb* and *rmpA*. Furthermore, these hvKp isolates were hypermucoviscous, confirmed by a positive string test (Pichler et al., 2017; Wahl et al., 2024). Characteristics

of the above mentioned six isolates and further isolates used to determine serum resistance and growth in platelet concentrates are given in Table 1. The bacterial species was determined by an automated system (Vitek 2 ID card, bioMérieux, Nuertingen, Germany); and antimicrobial susceptibility testing (ampicillin, cefotaxime, ceftazidime, meropenem) was done using broth microdilution with result interpretation according to the criteria of EUCAST version v14.0 (EUCAST 2024).

DNA and plasmid isolation, PCR and sequencing

Genomic DNA was isolated by various methods for PCR and Sanger sequencing (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany; MagAttract HMW DNA Kit, Machery & Nagel, Germany). Presence of beta-lactamase genes (*bla*_{TEM-like}, *bla*_{SHV-like}, *bla*_{CTX-M-Group1&9}, *bla*_{NDM-like}, *bla*_{OXA-48-like}, *bla*_{OXA-1-like}, *bla*_{OXA-9-like}) and virulence genes (*rmpA/A2*, *magA*) was determined using PCR and Sanger sequencing as previously described (Wahl et al., 2024). For whole genome sequencing (WGS), isolates were grown in Brain Heart Infusion (BHI) broth (BD, Heidelberg, Germany). DNA was extracted from overnight cultures using DNeasy Blood and Tissue Kit (Qiagen). DNA was quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The sequencing libraries were prepared using a Nextera XT DNA Library Prep Kit (Illumina®, San Diego, CA, USA). Sequencing was performed according to the manufacturer's protocol on an Illumina Miseq using v3 chemistry (2 × 300 bp) according to the manufacturer's protocol. Raw WGS data were processed as described (Wahl et al., 2024). Capsule types and virulence scores were determined using tools like Kleborate (v3.1.2) and Kaptive (Wyres et al., 2020; Lam et al., 2021; Lam et al., 2022). Kleborate is a bioinformatic tool for *K. pneumoniae* allowing species identification, MLST typing and resistance and virulence gene prediction from genomes. Kaptive allows K-locus (capsule) and O-locus (lipopolysaccharide) classification from genome data.

Cell lines used for cell biological assays

A549 human lung cells (ATCC CCL185, LGC Standards) were cultured in RPMI (Biowest, Nuaille, France) medium supplemented with 10% FCS. HT29-MTX human colonic epithelial cells were grown in DMEM medium (high glucose, stable glutamine, sodium pyruvate) (Biowest) supplemented with 10% FCS and non-essential amino acids (Biowest). HuTu80 (Cell Line Service) human duodenum cells were kept in DMEM (Biowest) medium (high glucose, stable glutamine, sodium pyruvate) supplemented with 10% FCS. RAW246.7 murine macrophages (ATCC TIB-71™) were cultured in RPMI (sodium pyruvate) (Biowest) supplemented with 10% FCS. 100 U/mL penicillin and 100 µg/mL streptomycin (Biowest) were added to each medium. Cultures were then incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

TABLE 1 *Klebsiella pneumoniae* isolates used for this study (grouped by pathotype).

Designation	Origin	Beta-lactam resistances	Beta-lactamase*	MLST type	Capsule type	O-locus	Virulence genes*	String test	Virulence score*	Pathotype category	Reference
17-0683 ¹	H: blood culture	AMP	SHV-11	ST1754	KL62	O1/O2v1	-	-	0	cKp	This study
17-0684 ¹	H: blood culture	AMP	SHV-108	ST889	KL54	O1/O2v1	ybt	-	1	cKp	This study
18-0014	A: bovine mastitis	AMP	SHV-116	ST609	KL114	O1/O2v1	-	-	0	cKp	This study
18-0018	A: bovine mastitis	AMP	SHV-11	ST403	KL23	O1/O2v2	-	+	0	cKp	This study
18-0030 ²	A: bovine mastitis	AMP	SHV-116	ST310	KL1	O1/O2v1	-	-	0	cKp	This study
ATCC 13883 ²	unknown	AMP	SHV-11	ST395	K3	O1/O2v1	ybt,rmpA,clb	-	2	cKp	Reference strain
PEI-B-P-08 ²	H: invasive isolate	AMP	SHV-11	ST48	K62	O1/O2v1	ybt	-	1	cKp	Reference strain (Spindler-Raffel et al., 2017)
16-0382 ¹	H: urine sample	AMP, CTX, CAZ	CTX-M-15, SHV-28, OXA-1	ST15	KL24	O1/O2v1	ybt	-	1	ESBL-cKp	(Becker et al., 2018)
16-0383 ¹	H: throat swab	AMP, CTX, CAZ	CTX-M-15, SHV-83, OXA-1	ST29	KL30	O1/O2v2	-	+	0	ESBL-cKp	(Becker et al., 2018)
18-0414 ²	H: unknown	AMP, CTX, CAZ, MER	SHV-11, CTX-M-15, OXA-48, OXA-1, TEM-1	ST392	K27	O4	ybt,rmpA2	-	1	ESBL-cKp	(Wahl et al., 2024)
ATCC 700721 ²	H: sputum	AMP	SHV-11 + 12, OXA-9, TEM-1	ST38	K52	OL13	-	-	0	ESBL-cKp	Reference strain
17-0609 ^{1,2}	H: liver abscess	-	-	ST2398	KL2	O1/O2v2	ybt,iro,iuc, clb,rmpA	+	5	hvKp	(Pichler et al., 2017)
18-0005 ¹	H: throat swab	-	-	ST66	KL2	O1/O2v2	ybt,iro,iuc, clb,rmpA	+	5	hvKp	(Klaper et al., 2021)
19-0213 ²	H: liver abscess	AMP, CTX, CAZ, MER	SHV-1, CTX-M-55, NDM-1, OXA-48	ST23	K57	O1/O2v1	ybt,iuc, rmpA,rmpA2	+	4	hvKp	(Wahl et al., 2024)
17-0736 ²	H: blood culture	AMP	SHV-11	ST5	KL39	O1/O2v1	ybt,iro, iuc,rmpA	+	4	hvKp	(Wahl et al., 2024)
19-0036 ²	H: blood culture	AMP	SHV-1	ST412	K57	O3b	iro,iuc, rmpA,rmpA2	-	3	hvKp	(Wahl et al., 2024)
NCTC 14052 ²	H: liver abscess	AMP	SHV-11	ST23	KL1	O1/O2v2	ybt,iro,iuc,clb, rmpA,rmpA2	+	5	hvKp	Reference strain

Kleborate v3.1.2 was used to determine MLST, capsule type, O-locus, virulence genes and score. AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; MER, meropenem; cKp, classical *K. pneumoniae*; ESBL-cKp, ESBL producing cKp; hvKp, hypervirulent *K. pneumoniae* [virulence score ≥ 3 or virulence score 2 plus positive string test and/or *rmpA* and/or *rmpA2*, see (Wahl et al., 2024)]; H, human; A, animal; ybt, yersiniabactin; iro, salmochelin; iuc, aerobactin; clb, colibactin; *rmpA/rmpA2*, regulators of the mucoid phenotype A and A2; * resistance gene prediction and virulence score according to the latest version of Kleborate v3.1.2 (<https://github.com/klebgonomics/Kleborate/wiki/Scores-and-counts>); MLST, multilocus sequence type; ¹, strains used in assays of adhesion, invasion, replication and cytotoxicity; ², strains used in investigations of serum resistance and growth in platelet concentrates.

Adhesion assay

A549 cells and HT29-MTX cells were seeded in 96-well plates (Greiner bio-one Cellstar) at a density of 3×10^3 cells per well, respectively. The cells were allowed to grow and differentiate for 24 hours at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. Overnight cultures of the bacterial strains to be analyzed were adjusted in PBS to an OD₆₀₀ of 0.2 (2×10^8 bacterial cells). An inoculum corresponding to a multiplicity of infection (MOI) of 100 (A549) or 25 (HT29-MTX) was prepared in DMEM and used to infect the cells. For the infections, 100 µl of medium was removed from the cells and replaced with 100 µl of the inoculum. After a 5-minute centrifugation step at 500 rpm, the bacteria adhered during a 1 h incubation at 37°C and 5% CO₂. The culture medium was then removed and non-adherent cells were removed by washing twice with PBS. The cells were lysed with PBS containing 1% Elugent (Merck Millipore, Darmstadt, Germany) and 0.0625% Antifoam B (Sigma-Aldrich, Schnelldorf, Germany) by incubating on a plate rocker at 800 rpm for 20 min at 24°C. 100 µl PBS was added to the lysates and a dilution series (several 10x dilutions) was prepared in PBS, of which 50 µl were plated on MH agar plates. The inoculated agar plates were then incubated overnight at 37°C.

Invasion assays

A549 and HuTu80 cells were seeded in 96-well plates at a density of 3×10^3 cells per well, respectively. To determine the invasion rate of *K. pneumoniae* isolates, the cell lines were infected in microtiter plates as described above using a MOI of 100 (A549) or 50 (HuTu80). Cells were washed with PBS after infection and incubated for 1 h in cell culture medium with 100 µg/ml amikacin at 37°C and 5% CO₂ to kill extracellular bacteria. After 1 h, the extracellular bacteria were removed by washing with PBS and the cells were lysed, diluted (several 10x dilutions) and plated as described above.

Phagocytosis and replication assays

RAW264.7 cells (ATCC TIB-71™) were seeded in 96-well plates at a density of 3×10^4 cells per well. To investigate the phagocytosis rate and intracellular replication of *K. pneumoniae*, cells in two microtiter plates were infected in parallel using the desired MOI of 5. The phagocytosis assay was conducted as described for invasion assays. In the second microtiter plate for the intracellular replication assay, the media was replaced with new RPMI with 10 µg/ml amikacin and incubated for 24 h. The cells were then lysed as described above and dilution series (several 10x dilutions) were plated to determine the replication rate using the invasion assay as a reference.

Cytotoxicity assays

To investigate a cytotoxic effect on intestinal cells, HuTu80 cells were infected with *K. pneumoniae* isolates as described above.

Deviating from this, after killing and removal of extracellular bacteria, live/dead staining of the eukaryotic cells was performed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Thermo Fischer Inc) according to the manufacturer's protocol.

Growth behavior in platelet concentrates

Ten *K. pneumoniae* strains (see Table 1) were examined with regard to their growth behavior in platelet concentrates. For this purpose, the *K. pneumoniae* strains were grown overnight in LB medium at 37°C with shaking. On the following day, subcultures were inoculated in LB medium with an OD₆₀₀ of 0.1 and incubated up to an OD₆₀₀ of 0.3 at 37°C and 120 rpm. A dilution series of up to 1:100,000 in 0.85% NaCl solution was then prepared from the bacterial suspensions, which corresponds to a cell count of approximately 10 CFU (colony forming unit)/ml. To determine the inoculum, the 10⁻⁵ dilution, with which the platelet concentrates were later spiked, was plated in triplicates on LB agar plates using a spiral plater (Eddy Jet, Neutec Group, Farmingdale, NY) and incubated overnight at 37°C. Buffy-coat derived pooled platelet concentrates (German Red Cross, Frankfurt, Germany) were pooled in a sterile beaker. As a sterile control, 10 ml of the concentrates were transferred to blood culture bottles and incubated for seven days in the BacT/ALERT system (bioMérieux, Nürtingen, Germany). Furthermore, the platelet concentrates were transferred to 25 ml blood bags, spiked with 10 CFU of the *K. pneumoniae* isolates to be analyzed and incubated for 43 h at 22.5°C with shaking. After the first 10 h, samples were taken every 3 h, diluted and plated in triplicates with a spiral platter on LB agar plates and incubated overnight at 37°C. The last sample was taken after a total of 43 h. The bacterial colonies were counted automatically using the Sphere Flash apparatus (Neutec Group, Farmingdale, NY).

Serum resistance assays

In this study, ten *K. pneumoniae* strains (see Table 1) were analyzed with regard to their resistance to human serum. For this purpose, the *K. pneumoniae* strains were cultivated overnight at 37°C in LB medium with shaking. On the following day, subcultures were inoculated in LB medium with an OD₆₀₀ of 0.1 and incubated up to an OD₆₀₀ of 0.3 at 37°C and 120 rpm. From the bacterial cultures, 2 ml were pelleted for 5 min at 10000 rpm. The cell pellet was washed with 0.85% NaCl solution, pelleted again for 5 min at 10,000 rpm, and the cell pellet was taken up in 1 ml of 0.85% NaCl solution, which corresponds to a cell count of approximately 2×10^8 CFU/ml. A dilution series up to 1:100,000 was then prepared from the bacterial suspensions in PAS-II (115.5 mM/l NaCl; 10 mM/l Na-Citrate; 30 mM/l Na-Acetate), corresponding to a cell count of approximately 2×10^3 CFU/ml. To determine the inoculum, the 10⁻⁵ dilution, with which the human serum was inoculated, was plated in triplicates on LB medium using a spiral platter and incubated overnight at 37°C. Furthermore, 500 µl of the 10⁻⁵ dilution was

mixed with 500 μ l of 50% human serum (Merck, Darmstadt, Germany), which corresponds to a cell count of 10^3 CFU/ml and a serum concentration of 25% (as in platelet concentrates). The preparations were incubated in a thermoshaker at 37°C and 400 rpm (Eppendorf Thermomixer® C). After 1 h and 4 h, 100 μ l of the cultures were plated in triplicates with using a spiral plater on LB agar plates and incubated overnight at 37°C. The next day, the bacterial colonies were automatically counted using the Sphere Flash apparatus.

Light microscopy

For microscopy of the cell morphology of the different *K. pneumoniae* pathotypes (*cKp*, ESBL-*cKp*, *hvKp*), various isolates were cultivated at 37°C until the stationary growth phase (isolates 16-0382, 16-0383, 17-0683, 17-0684, 17-0609, 18-0005; see Table 1). One ml of the bacterial culture was centrifuged at 14,000 rpm for 5 min. The cell pellet was resuspended in 1 ml crystal violet solution (50 μ l crystal violet + 950 μ l PBS) and incubated for 1 min. The culture was then centrifuged again and the cell pellet was washed in PBS. A 60% ink solution (in PBS) was added to the bacterial suspension at a ratio of 1:2. 0.2 μ l of the ink stained suspension was added to a slide coated with 1.5% agarose (in PBS) and covered with a coverslip. The phase contrast of the Nikon Eclipse Ti microscope was used to analyze the cell morphology of the bacterial cells under a 100 $\times$ oil immersion objective and the images were recorded using a Nikon DS-MBWc CCD camera. To

determine the cell lengths, 100 bacterial cells per strain were measured in triplicates using the MicrobeJ program. MicrobeJ is a software plugin tool developed for ImageJ to detect and count bacterial cells (<https://www.microbej.com/>; last access 08.01.2025).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism or R software. Unless otherwise specified, all analyses were performed based on three independent experiments. The results were presented as mean $\pm$ standard deviation (S.D.). Detailed information regarding the specific statistical tests employed for each analysis can be found in the corresponding figure legends.

Results

Cell morphological differences of *K. pneumoniae* pathotypes

The six clinical *K. pneumoniae* isolates 16-0382, 16-0383, 17-0683, 17-0684, 17-0609, 18-0005 (see Table 1) were analyzed for possible cell morphological differences using ink staining and light microscopy. Based on the microscopic images, the thick capsular layer known for *hvKp* was observed and appears as a clear white halo around the bacterial cells (Figure 1A). In addition, clear differences in the cell length of the *hvKp* in comparison to the

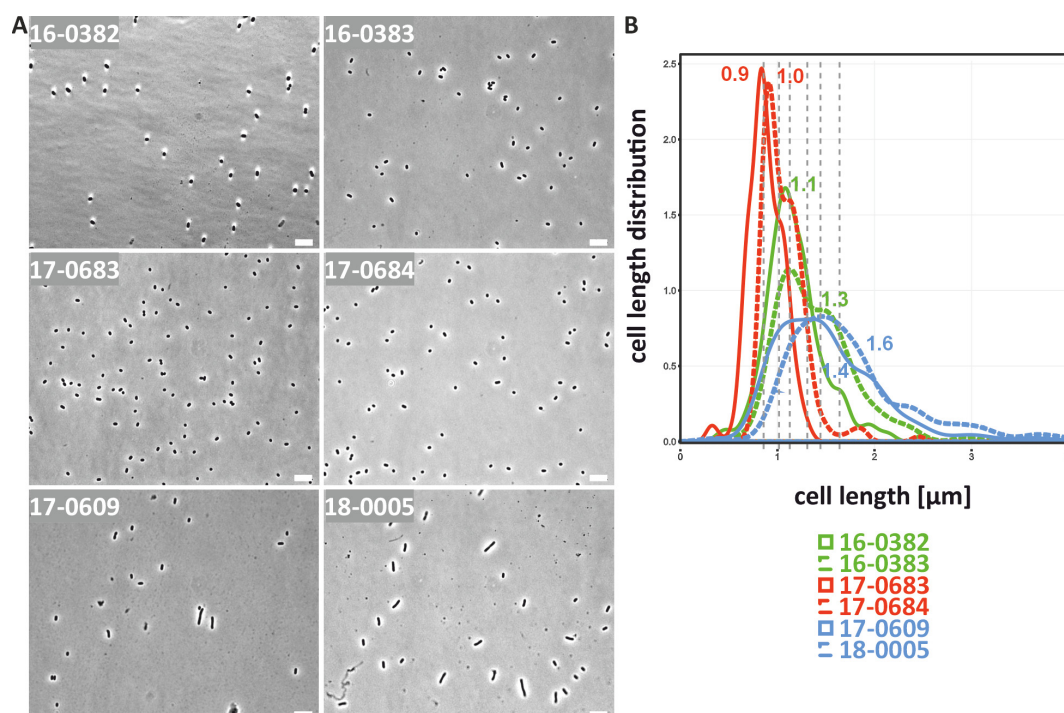


FIGURE 1

Cell morphological differences of *cKp*, ESBL-*cKp* and *hvKp* isolates under normoxia. (A) Light microscopic images of *cKp* (17-0683 and 17-0684), ESBL-*cKp* (16-0382 and 16-0383) and *hvKp* isolates (17-0609 and 18-0005) showed a difference in capsule thickness and cell length. (B) The distribution of cell lengths of the analyzed *K. pneumoniae* isolates is shown. Using MicrobeJ, triplicates of each strain with 100 cells each were measured, analyzed with R and visualized.

cKp and the ESBL-*cKp* isolates were observed. As can be seen in Figure 1, the cells of the *hvKp* isolates are longer and have a filamentous shape, while the *cKp* and ESBL-*cKp* isolates have a rod shape typical of bacteria of the *Enterobacterales* family. In order to quantify this result, the cell lengths distribution was analyzed as visualized in microscopic images. This revealed that the median cell length of *hvKp* isolates is longer than that of *cKp* and ESBL-*cKp* isolates. In addition, the *hvKp* isolate 18-0005 had longer cells than *hvKp* isolate 17-609.

Cell morphological changes of *K. pneumoniae* pathotypes under hypoxia

K. pneumoniae is a facultative anaerobic bacterium that grows optimally in the presence of oxygen, but can also survive in low-oxygen environments such as the intestine. For *E. coli*, it has already been shown that they react to oxygen reduction by filamentation of the bacterial cells and that this has an influence on the pathogenicity (Murashko and Lin-Chao, 2017). It should therefore be investigated whether cell morphological changes in *K. pneumoniae* can also be observed under oxygen reduction. For this purpose, the isolates were cultivated overnight at an oxygen concentration of 0.5 vol% and analyzed microscopically. As shown in Figure 2, an elongation of the bacterial cells was observed in light microscopic images and cell length measurements for representatives isolates of all three pathotypes. As previously shown under normoxia, the bacterial cells of the *hvKp* isolates were longer than those of the *cKp* and

ESBL-*cKp* isolates. The results show an altered cell length of *K. pneumoniae* depending on oxygen. Due to the similarity to *E. coli* in the O₂-dependent length distribution, we also suspect an influence of O₂ on the pathogenic potential of *K. pneumoniae*.

In vitro adhesion experiments

Studies have shown that elongated bacterial cells can adhere better to epithelial cells *in vitro* due to their enlarged cell surface (Yang et al., 2016). As described above, a filamentous form was observed for the bacterial cells of *hvKp* isolates 17-0609 and 18-0005. In the following, it was investigated whether a difference in adhesion can be observed between the different *K. pneumoniae* pathotypes. Because *K. pneumoniae* colonizes the respiratory and intestinal tracts, the lung cell line A549 and the intestinal cell line HT29-MTX were selected as *in vitro* cell models. Figure 3 shows the adhesion rates as a percentage of the inoculum. The *K. pneumoniae* isolates analyzed adhered to a lesser extent to A549 lung cells *in vitro* than to HT29-MTX intestinal cells. The adhesion rate was below 5% (Figure 3A). Furthermore, no differences in adhesion capacity were observed between the different *K. pneumoniae* pathotypes on A549 lung cells. Although the standard deviation was large due to fluctuating adhesion rates in the individual experiments, a trend could be recognized from the data showing a better adhesion of *cKp* cells (independent from the ESBL status) than *hvKp* cells (Figure 3B). The analyzed *cKp* and ESBL-*cKp* isolates showed a higher adhesion rate (about 10% of the inoculum) to intestinal cells than

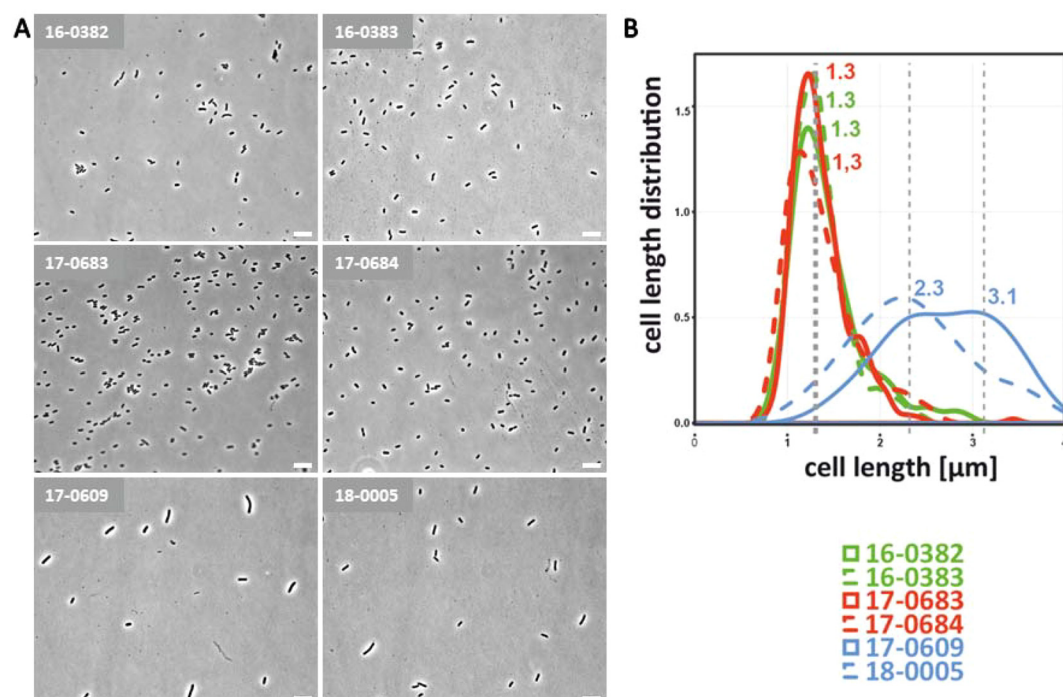


FIGURE 2

Cell morphological differences of *cKp*, ESBL-*cKp* and *hvKp* isolates under hypoxia. (A) Light microscopic images of *cKp* (17-0683 and 17-0684), ESBL-*cKp* (16-0382 and 16-0383) and *hvKp* isolates (17-0609 and 18-0005) showed a difference in capsule thickness and cell length. (B) Distribution of cell lengths of analyzed *K. pneumoniae* isolates. Using MicrobeJ, triplicates of each strain with 100 cells each were measured, analyzed with R and visualized.

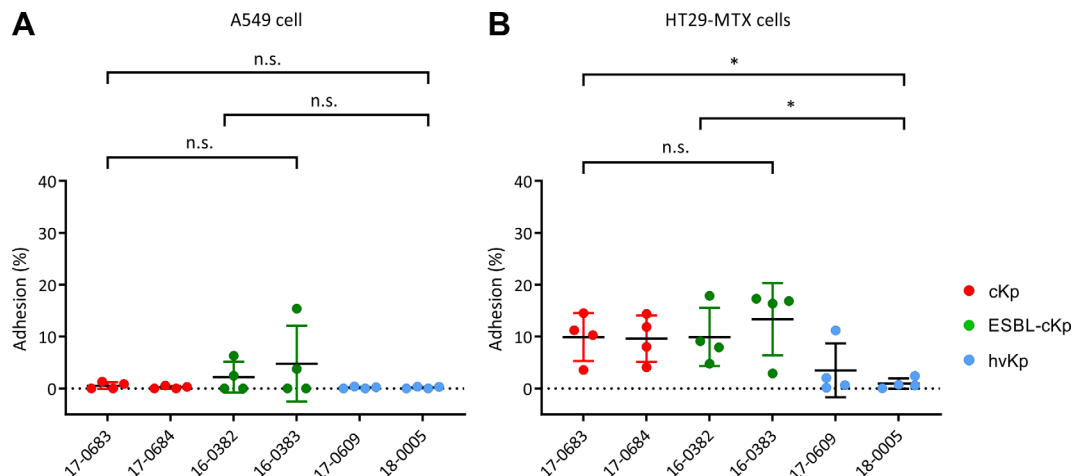


FIGURE 3

Quantification of the adherence of *K. pneumoniae* pathotypes to A549 and HT29-MTX cells. The adherence of cKp (17-0683 and 17-0684), ESBL-cKp (16-0382 and 16-0383) and hvKp isolates (17-0609 and 18-0005) to (A) A549 lung cells and (B) HT29-MTX intestinal cells is shown as a percentage of the inoculum. The data represent the results from four biologically independent triplicates. ANOVA was applied to the data to calculate significance between groups. Statistical significance is indicated as follows: n.s., not significant, * $P \leq 0.05$.

to lung cells in the *in vitro* cell culture model. In contrast, the adhesion rate of hvKp isolates was significantly lower, the adhesion rate here was also $< 5\%$. Thus, the results obtained in the infection experiments do not indicate a positive influence of cell morphology on the adhesion capacity.

In vitro cellular invasion assays

We investigated whether a difference in cellular invasion can be observed between the included representative isolates of the three *K. pneumoniae* pathotypes. To determine the cellular invasion, the lung cell line A549 and the intestinal cell line HuTu80 were selected

as models. Figure 4 shows the invasion rates as a percentage of the inoculum. The *K. pneumoniae* isolates analyzed showed very low invasion *in vitro* (invasion rate $< 1\%$) in A549 lung cells (Figure 4A) and HuTu80 intestinal cells (Figure 4B). As previously shown in the adhesion model, no significant differences were found between the different *K. pneumoniae* pathotypes with regard to invasion in A549 lung cells. However, differences were observed in the invasion rates determined in the cell culture model with HuTu80 intestinal cells. Here, the two ESBL-cKp isolates showed a significantly higher invasion rate than the two cKp and the two hvKp isolates. The results obtained with the infection models used did not show increased cellular invasion of the hvKp isolates compared to the cKp isolates analyzed.

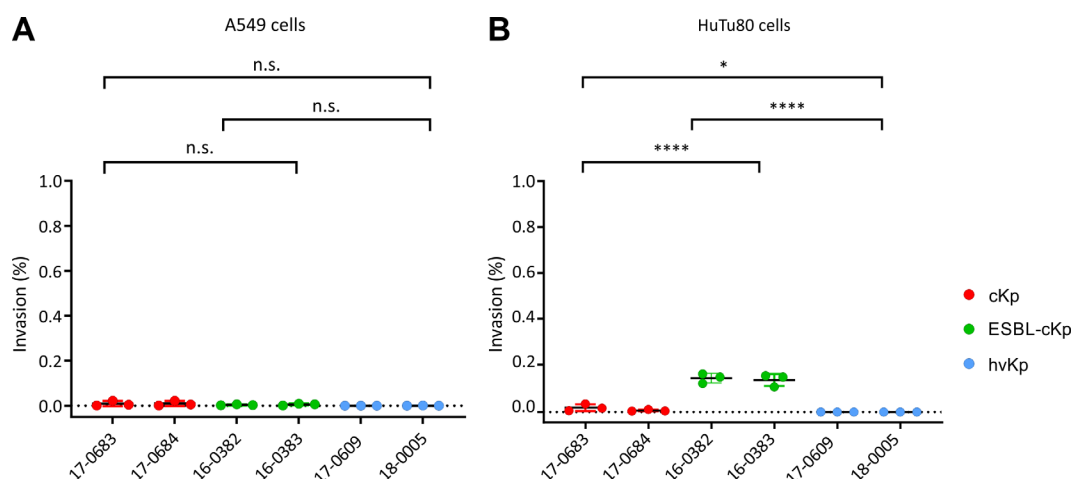


FIGURE 4

Quantification of the invasion of *K. pneumoniae* pathotypes in A549 and HuTu80 cells. The invasion of cKp (17-0683 and 17-0684), ESBL-cKp (16-0382 and 16-0383) and hvKp isolates (17-0609 and 18-0005) in (A) A549 lung cells and (B) HuTu80 intestinal cells in relation to the inoculum. The data represent the results from three biologically independent triplicates. ANOVA was applied to the data to calculate significance between groups. Statistical significance is indicated as follows: n.s., not significant, * $P \leq 0.05$, **** $P \leq 0.0001$.

In vitro phagocytosis experiments

Using an *in vitro* infection model with murine RAW 264.7 macrophages, the interaction of the *K. pneumoniae* isolates with phagocytes was determined. The aim was to characterize possible differences in phagocytosis resistance between the representative isolates of the different *K. pneumoniae* pathotypes. To this end, the phagocytosis rate of the six different *K. pneumoniae* strains was first compared. After a 1 h of infection, the number of intracellular bacteria was determined. The phagocytosis rate was calculated in relation to the inoculum. Figure 5A shows the phagocytosis rate of the six *K. pneumoniae* strains from three independent experiments. The hvKp isolates 17-0609 and 18-0005 showed a significantly lower phagocytosis rate *in vitro* compared to cKp and ESBL-cKp isolates (16-0382 and 16-0383).

To clarify whether the investigated *K. pneumoniae* strains survive and/or replicate intracellularly in RAW 264.7 macrophages, the number of intracellular bacterial cells was determined 24 h after infection and the replication rate was calculated in relation to the phagocytosis rate. The hvKp isolates 17-0609 and 18-0005 were killed within the macrophages as indicated by a replication rate of less than one (Figure 5B). A replication rate of about one for cKp isolates 18-0683 and 18-0684 indicated intracellular survival without replication in mouse macrophages. In contrast, both ESBL-cKp isolates showed *in vitro* replication rates of about 90-fold. They can persist and replicate intracellularly in macrophages.

Comparisons of intracellular replication of *K. pneumoniae* pathotypes under normoxia and hypoxia

For some enteropathogenic bacteria such as *Salmonella*, it has already been shown that a reduction in oxygen is associated with increased virulence of the pathogen (Marteyn et al., 2011). After filamentation of the *K. pneumoniae* cells under oxygen reduction has

been observed (Figure 2), the aim was to investigate whether reduced oxygen availability also has an influence on the interaction with host cells. For this purpose, mouse macrophages were infected in parallel in two cell culture plates. One plate was incubated as usual under normoxia ($O_2 = 20.9\%$) and the second plate under hypoxia ($O_2 = 0.5\%$) for 24 hours. Figure 6 shows the replication rates of the analyzed six *K. pneumoniae* isolates. No significant difference was found in the replication rates under hypoxia. Again, only the ESBL-cKp isolates were able to replicate, while cKp isolates persisted and hvKp isolates died. No difference in intracellular replication was observed comparing hypoxic and normoxic culture conditions of the individual strains.

Cytotoxic effects of clinical *K. pneumoniae* pathotypes

As described above, no cellular invasion of intestinal cells was observed for hvKp isolates. However, studies by Cano et al. had shown a cytotoxic effect of *K. pneumoniae* on epithelial cells (Cano et al., 2009). Therefore, we investigated whether the six *K. pneumoniae* isolates 16-0382, 16-0383, 17-0683, 17-0684, 17-0609 and 18-0005 have a cytotoxic effect on intestinal cells. For this purpose, HuTu80 intestinal cells were infected with the *K. pneumoniae* isolates as previously described. Live/dead staining with calcein AM (viable) and ethidium bromide (non-viable) was used to discriminate between viable and non-viable HuTu80 cells, which were analyzed both photometrically and microscopically. Figure 7 shows the percentages of viable and non-viable HuTu80 cells. All investigated cKp, ESBL-cKp and hvKp isolates showed a cytotoxic effect on intestinal cells *in vitro*. The viability of HuTu80 cells after infection was below 80% for isolates of all pathotypes and the percentage of non-viable cells was above 20%. HvKp isolates showed a stronger cytotoxic effect *in vitro* than cKp and ESBL-cKp isolates. Microscopic images of cells infected with hvKp isolates showed the loss of integrity of the HuTu80 monolayer, whereas a confluent cell lawn persisted after infections with cKp and ESBL-cKp isolates (Figure 7C).

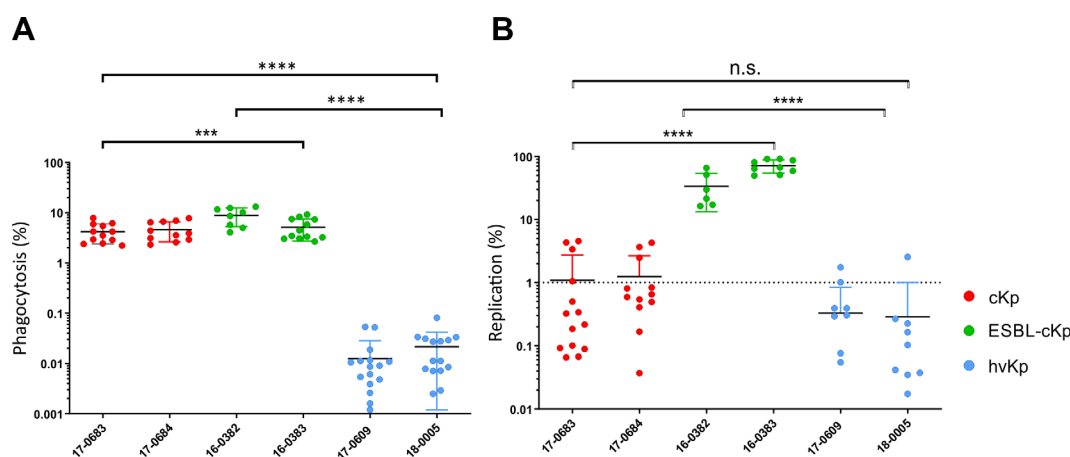


FIGURE 5

Quantification of phagocytosis resistance and intracellular replication of different *K. pneumoniae* pathotypes. (A) Phagocytosis rate of cKp (17-0683 and 17-0684), ESBL-cKp (16-0382 and 16-0383) and hvKp isolates (17-0609 and 18-0005) by murine RAW 264.7 macrophages (60,000 cells/cavity) after one hour of infection. (B) Replication rate of cKp (17-0683 and 17-0684), ESBL-cKp (16-0382 and 16-0383) and hvKp (17-0609 and 18-0005) isolates in murine RAW 264.7 macrophages (60,000 cells/cavity) after 24 hours of infection. ANOVA was applied to the data to calculate significance between groups. Statistical significance is indicated as follows: n.s., not significant, *** $P \leq 0.01$ and **** $P \leq 0.0001$.

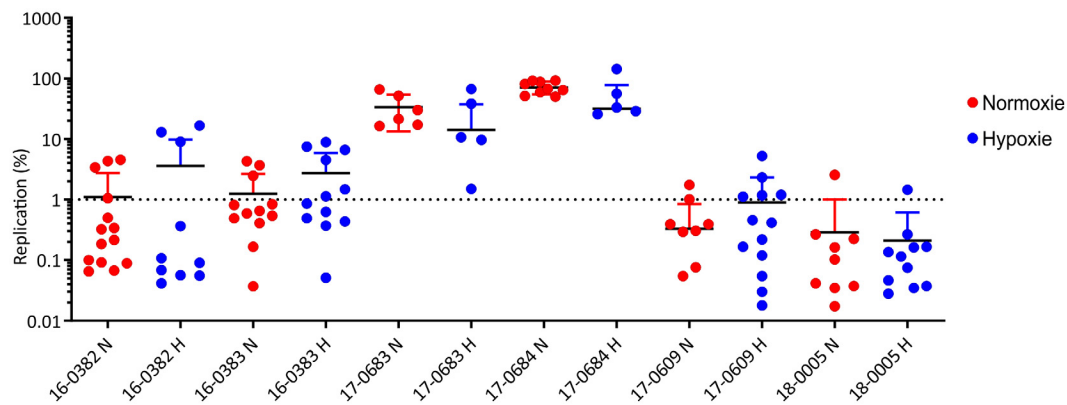


FIGURE 6

Comparison of the replication of *K. pneumoniae* isolates representing different pathotypes in RAW 264.7 mouse macrophages under normoxia (N, red) and hypoxia (H, blue). The replication rates of *cKp* (17-0683 and 17-0684), ESBL-*cKp* (16-0382 and 16-0383) and *hvKp* isolates (17-0609 and 18-0005) in murine RAW 264.7 macrophages (60,000 cells/cavity) under normoxia (N; O₂ = 20.9%; red) and hypoxia (H; O₂ = 0.5%; blue) are shown.

Growth of *K. pneumoniae* isolates in platelet concentrates

The growth behavior of 10 *K. pneumoniae* isolates, which harbor different siderophore genes (Table 1), was compared with that of the *K. pneumoniae* reference strain PEI-B-P-08 [143] in platelet concentrates. For this purpose, a 50 ml platelet concentrate

was spiked with 10 CFU of one of the *K. pneumoniae* isolates to be tested and incubated under conditions specified by haemotherapy guidelines (Spindler-Raffel et al., 2017; Prax et al., 2019). Figure 8 shows the growth kinetics of the analyzed *K. pneumoniae* isolates over a period of 43 hours. For reference strain PEI-B-P-08 (*cKp*), *hvKp* reference strain NCTC 14052 and test strains 18-0030 (*cKp*) and 17-0609 (*hvKp*), an increase in bacterial count was observed

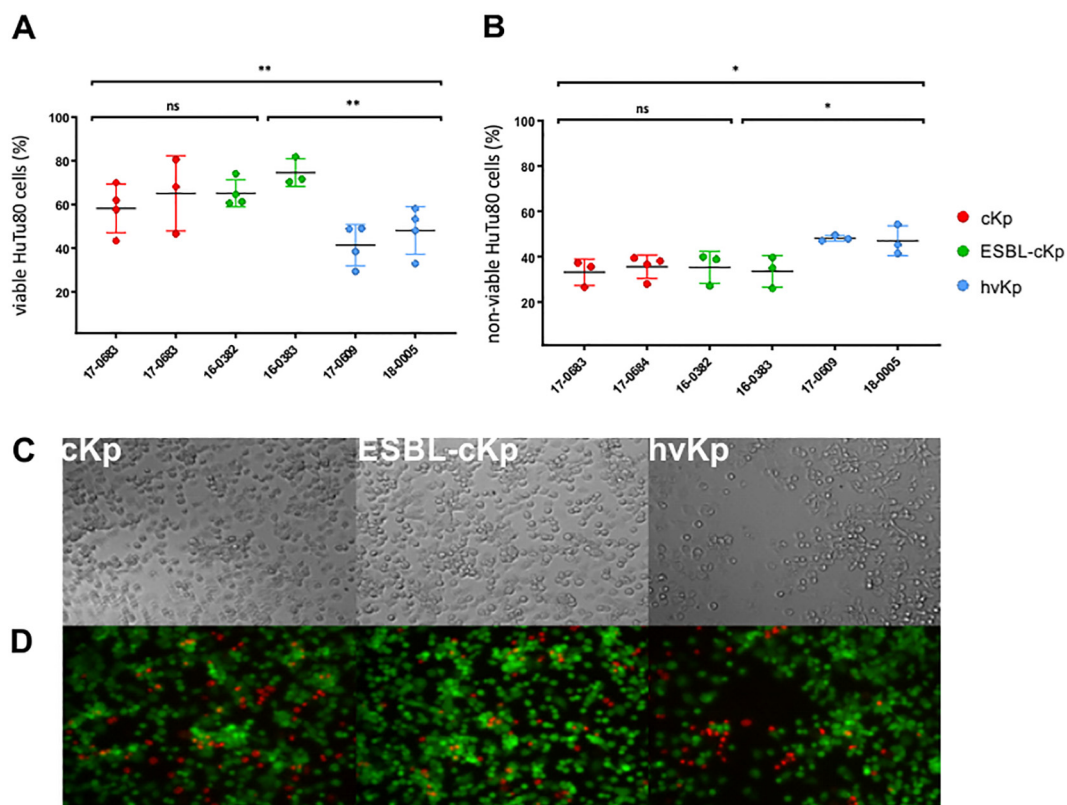


FIGURE 7

Comparison of the cytotoxic effect of *K. pneumoniae* pathotypes on HuTu80 intestinal cells, showing the percentage of viable (A) and non-viable (B) HuTu80 intestinal cells after infection with *cKp* (17-0683 and 17-0684), ESBL-*cKp* (16-0382 and 16-0383) and *hvKp* isolates (17-0609 and 18-0005). The data represent the results of triplicates from three independent experiments. ANOVA was applied to the data to calculate significance between groups. Statistical significance is indicated as follows: * $P \leq 0.05$ and ** $P \leq 0.01$. Transmitted light microscopy (C) and fluorescence microscopy (D) images of viable (green) and non-viable (red) HuTu80 intestinal cells after infection with *K. pneumoniae*. n.s., not significant.

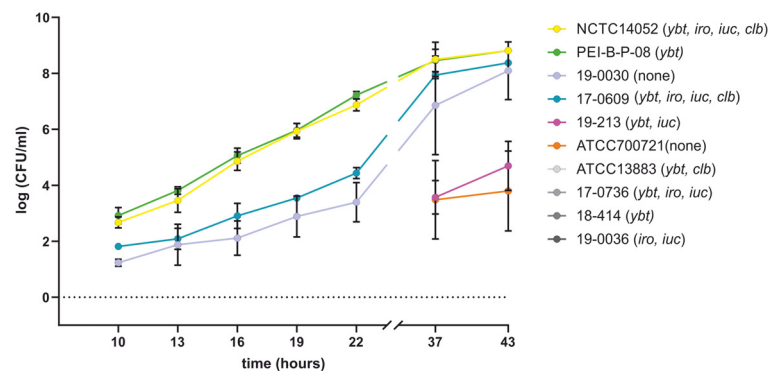


FIGURE 8

Comparison of the growth behavior of *K. pneumoniae* isolates in platelet concentrates. The growth curves of *K. pneumoniae* isolates ATCC 13883, ATCC 700721, NCTC 14052, PEI-B-P-08, 17-0609, 17-0736, 18-0030, 18-0414, 19-0036 and 19-0213 at 22.5°C in platelet concentrates are shown. No growth could be detected for the four isolates labelled with grey colors. The data represent the results of triplicates from three independent experiments.

after 10 h, with the bacterial count of isolates NCTC 14052 and PEI-B-P-08 being one order of magnitude higher than that of isolates 18-0030 and 17-0609. Furthermore, the growth of isolates NCTC 14052 and PEI-B-P-08 was exponential, while the growth of isolates 18-0030 and 17-0609 showed a linear increase. After 37–43 h, the growth of isolates NCTC 14052, PEI-B-P-08, 18-0030 and 17-0609 was in the stationary phase with bacterial counts of about 10^7 . The isolates 19-0213 (hvKp) and ATCC 700721 (cKp) exhibited a long lag phase of over 22 h. Only after 38 h a significant increase in bacterial counts was detected for these isolates. Four further isolates analyzed (ATCC13883 (cKp), 17-0736 (hvKp), 18-0414 (ESBL-cKp) and 19-0036 (hvKp) were unable to grow in platelet concentrates. This growth behavior could be due to different serum resistances (Doorduyn et al., 2016).

Serum resistance of *K. pneumoniae* isolates

In order to investigate possible serum resistance of the ten *K. pneumoniae* isolates, human serum was inoculated with 10^3 CFU of

the isolates to be analyzed. Figure 9 shows the results of the bacterial counts determined after 1 h and 4 h. The isolates PEI-B-P-08 (cKp), NCTC 14052 (hvKp), 17-0609 (hvKp), 18-0030 (cKp), 19-0213 (hvKp) and ATCC 700721 (cKp), were resistant to human serum. While the bacterial counts of isolates ATCC 700721 remained stable, increased bacterial counts were observed for isolates NCTC 14052, PEI-B-P-08, 18-0030, 17-0609, and 19-0213. The isolates ATCC 13883 (cKp), 17-0736 (hvKp), 18-0414 (ESBL-cKp) and 19-0036 (hvKp) are not resistant to human serum, here bacterial counts decreased over 4 h.

Discussion

Previous studies have focused on genome-wide association studies of globally distributed *K. pneumoniae* lineages (“high-risk clones”) and epidemiological studies to identify risk factors that favor nosocomial *K. pneumoniae* infections (Cano et al., 2009; Meatherall et al., 2009; Martin and Bachman, 2018; Cienfuegos-Gallet et al., 2019). However, in the face of increasing antibiotic

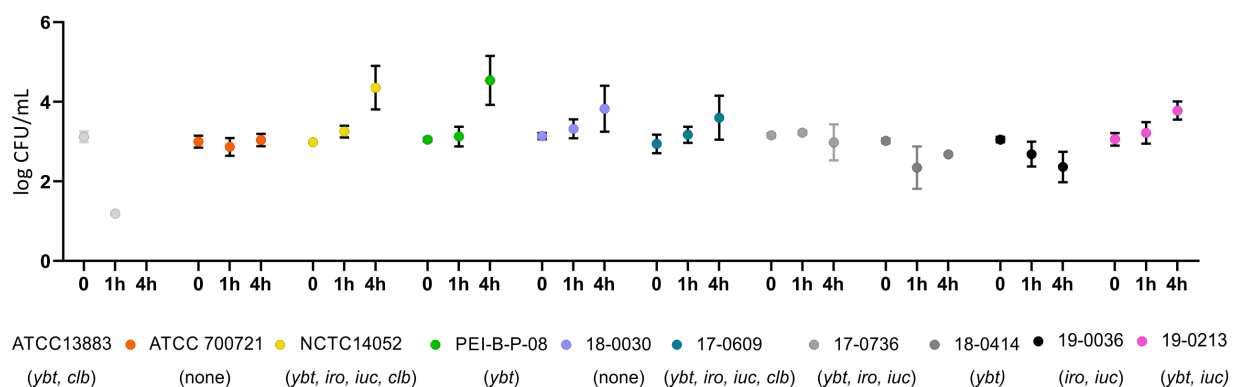


FIGURE 9

Comparison of the serum resistance of ten *K. pneumoniae* isolates. Serum resistance of *K. pneumoniae* isolates ATCC 13883, ATCC 700721, NCTC 14052, PEI-B-P-08, 17-0609, 17-0736, 18-0030, 18-0414, 19-0036 and 19-0213 at 37°C in 25% human serum. No growth could be detected for isolates labelled in different grey colors. The data represent the results of triplicates from three independent experiments.

resistance and the global spread of hypervirulent strains, a better understanding of the pathogenesis of *K. pneumoniae* is essential for the development of preventive measures and more targeted treatments including “anti-virulence strategies”. Therefore, the present study investigated properties of different *K. pneumoniae* pathotypes in order to be able to make statements about host adaptations that contribute to the successful spread of this bacterium in the different habitats.

First, differences in the cell morphology of hvKp isolates compared to cKp and ESBL-cKp isolates were determined and documented by means of light microscopy (Figures 1, 2). The analyzed hvKp isolates showed a filamentous phenotype. In the literature, filamentation of bacterial cells is often described as the result of impaired cell division (Klein et al., 2015; Cayron et al., 2023). However, some bacteria are able to react to stress conditions such as oxidative stress, nutrient deficiency or the defense mechanisms of the host immune system by a temporary change in cell morphology (Justice et al., 2008). For *E. coli*, it has been observed that the enlargement of the cell surface associated with a filamentous phenotype has a positive effect on adhesion to surfaces and protects the bacteria from phagocytosis by immune cells (Klein et al., 2015). In our study, cell filamentation of hvKp was much more pronounced when compared to cKp and ESBL-cKp, but cell lengths’ variation among individual hvKp isolates was also remarkable suggesting a potential greater polymorphic population than among cKp and ESBL-cKp. However, adhesion of hvKp to two different cell lines was weaker compared to cKp (Figures 1–3). Thus, the positive effect of cell filamentation might be compensated by the protective effect of the larger capsule (Figure 3). Under hypoxia, an increase in cell length was also observed for cKp and ESBL-cKp isolates, but to a much lesser extent than for hvKp isolates. It is known that filamentous bacteria have an advantage in adherence to epithelial cells. As hypoxia conditions prevail in the gut, reduced oxygen levels could be a reason for *K. pneumoniae* to alter cell morphology in order to adhere better to the intestinal epithelium.

The cell culture model was used to investigate whether representative isolates of the three *K. pneumoniae* pathotypes show differences in the pathogen-host interaction. Since respiratory and gastrointestinal colonization of the bacterium is considered to be the main reservoir for *K. pneumoniae* infections, the cell infection experiments were carried out with the lung cell line A549 and the intestinal cell lines HT29-MTX and HuTu80 (Sun et al., 2019). In previous cell infection experiments, the adhesion and cellular invasion of encapsulated and non-encapsulated *K. pneumoniae* isolates were compared (Sahly et al., 2000; Cortes et al., 2002; Struve and Krogfelt, 2003; Opoku-Temeng et al., 2019). A direct correlation between the adhesion to and invasion of lung epithelial cells and the ability of *K. pneumoniae* isolates to express capsular polysaccharide has been observed (Fumagalli et al., 1997; Sahly et al., 2000; Struve and Krogfelt, 2003; de Astorza et al., 2004). No differences of the included representative strains representing the three *K. pneumoniae* pathotypes cKp, ESBL-cKp and hvKp in adherence to and invasion of A549 lung cells were found (Figures 3A, 4A). In general, adhesion/invasion rates are lower with the lung cell lines compared to intestinal cells. These results suggest that adhesion and invasion of lung cells is not a

classical pathophysiological mechanism of *K. pneumoniae*. One possible way in which the bacterium is able to overcome the cell barrier in the respiratory tract is by disrupting the cell integrity of the epithelium through injury in the respiratory tract or, as described by Cano et al., via a cytotoxic effect on A549 lung cells by encapsulated *K. pneumoniae* isolates (Cano et al., 2009).

In the intestinal cell model, however, differences in adherence and invasion between the isolates representing the three different *K. pneumoniae* pathotypes were observed (Figures 3B, 4B). While the cKp and ESBL-cKp isolates are equally capable of adhering to intestinal cells, no adhesion was observed for the hvKp isolates analyzed. These results are congruent with the observations that a thick capsular layer reduces the adhesion capacity of *K. pneumoniae* (Sahly et al., 2000). Furthermore, the hypothesis that an enlarged cell surface increases adhesion due to a filamentous phenotype was not confirmed under normoxic conditions. Hus et al. were able to show in a cell culture model that *K. pneumoniae* can overcome the barrier function of the intestinal epithelium via a transcellular pathway (Hsu et al., 2015). This could only be confirmed for ESBL-cKp in the present study. The cKp and hvKp isolates showed no cellular invasion *in vitro* in the intestinal cell line used. These results are comparable to previous data by Sahly et al. who observed an increased cellular invasion of ESBL-producing *K. pneumoniae* isolates (Sahly et al., 2008), an effect that might potentially derive from additional features encoded on ESBL-containing plasmids than from the pure presence of the ESBL gene itself.

The clinical pictures of hvKp infections include invasive and necrotizing infections, which suggests a cytotoxic effect of hvKp isolates (Evangelista et al., 2018). Our study showed that the investigated hypervirulent isolates had a significantly higher cytotoxic effect on intestinal epithelial cells than cKp or ESBL-cKp isolates. Holes in the monolayer were observed after infection demonstrating the loss of epithelial integrity (Figure 7C). It can be assumed that the proportion of non-viable cells was even higher, as the detached (and subsequently washed away) cells could not be detected. This indicates that hvKp do not invade intestinal cells but (Figure 4), due to their cytotoxic effect, damage the intestinal epithelium and overcome the intestinal barrier in this way. Recent studies in particular describe virulence factors that support this hypothesis. For example, a genotoxic effect on eukaryotic cells was observed *in vitro* for colibactin-producing *K. pneumoniae* isolates (Lai et al., 2014; Lu et al., 2017). It is assumed that colibactin leads to apoptosis of intestinal cells and thus enables the intestinal translocation of hvKp (Secher et al., 2015; Wyres et al., 2020).

The capsule of *K. pneumoniae* is the most important virulence factor (Huynh et al., 2017; Opoku-Temeng et al., 2019). It protects the bacteria from antibiotics, the complement system of the immune defense and phagocytosis (Kabha et al., 1995). It is known from the literature that encapsulated *K. pneumoniae* are resistant to opsonization *in vivo* and to uptake by phagocytes (Domenico et al., 1994; Alvarez et al., 2000). The studies also compared encapsulated *K. pneumoniae* isolates and capsule mutants with each other. However, only the general role of the capsule on host interaction was investigated. No conclusions can be drawn about differences between the *K. pneumoniae* pathotypes.

The present study investigated the extent to which the abilities of phagocytosis resistance, intracellular persistence and replication differ between the included isolates representing the various *K. pneumoniae* pathotypes. Using a mouse macrophage infection model, significant differences were observed, both *cKp* and ESBL-*cKp* were equally phagocytosed by the immune cells, while the *hvKp* isolates were not (Figure 5).

Our results also showed no intracellular persistence or replication for *hvKp* isolates (Figure 6). There are several possible reasons for this. Firstly, increased capsule synthesis is characteristic of *hvKp* isolates, which makes them less accessible to macrophages. Furthermore, as described above, filamentation of the *hvKp* bacterial cells was observed. For some bacteria it is known that they change from the bacillary to the filamentous cell form and are therefore less accessible for phagocytotic cells (Prashar et al., 2013). However, the comparatively low number of phagocytosed *hvKp* must be considered as a limitation, which led to a higher variability of the individual experiments despite a large number of replicates.

The ability of ESBL-*cKp* to increase intracellular persistence compared to *cKp* as described by Cano et al. was confirmed in the present study (Cano et al., 2015) (Figures 5, 6). Interestingly, the ESBL-*Kp* isolates analyzed were also able to replicate in RAW macrophages. While *hvKp* were able to evade macrophage access, ESBL-*cKp* have found a strategy to replicate in macrophages similar to classical intracellular pathogens such as *Legionella pneumophila* and *Salmonella enterica* (Price and Vance, 2014).

There is currently a lack of a standardized method for virulence prediction of *K. pneumoniae* and thus a derivation of markers for laboratory diagnostics. To date, virulence analyses have concentrated on genome comparisons and genome-wide association studies (Spadar et al., 2022; Cheung et al., 2023). A conventional assessment of the virulence potential and the differentiation of *cKp* and *hvKp* isolates is currently under discussion and is based, among other things, on the detection of specific iron transport systems (Holt et al., 2015; Lam et al., 2018b; Lam et al., 2018a). Molecular epidemiological studies have shown that the prevalence of iron transport systems is higher in *hvKp* isolates than in *cKp* isolates (Russo et al., 2015; Russo and Marr, 2019).

In this study, the growth of *hvKp* isolates with different siderophore systems (Table 1) was compared with that of the reference strain PEI-BP-08 in platelet concentrates. The PEI-BP-08 reference isolate (ST48-K62), which only possesses *ybt*, and the *hvKp* reference strain NCTC 14052 (ST23-K1) harboring all relevant siderophore genes (*ybt*, *iro*, *iuc*, *clb*) and capsule regulator genes (*rmpA/rmpA2*), in addition, were among the fast-growing isolates. *cKp* isolate 18-0030 (ST310-K1) and *hvKp* isolate 17-0609 (ST3895-K2) were also able to proliferate in platelet concentrates, but their proliferation was delayed. Interestingly, for the *hvKp* isolate 19-0213 (ST23-KL107) and ESBL-*cKp* isolate ATCC 700721 (ST38-K15), proliferation was only detected after an incubation period of 37 hours. Bacteria with such a long lag phase represent a major challenge in transfusion medicine, as such bacterial contamination is difficult to detect and harbors the risk of transfusion-related bacterial infections. Despite the presence of siderophores, no growth could be observed for four of the analyzed isolates of various classifications and with presence of several siderophore genes.

In contrast to the present knowledge and working hypotheses (Holt et al., 2015; Russo and Marr, 2019), our study results indicate

that siderophores are not essential factors for the proliferation of *K. pneumoniae* in blood components. It was shown that virulence prediction by the presence or absence of siderophores alone is not necessarily associated with the ability to proliferate in blood components, which calls into question the reliability of the proposed classification of *hvKp* isolates based on the presence of specific iron transport systems only (Holt et al., 2015; Lam et al., 2021). Instead, the results of the present work provide evidence that the capsule plays the decisive role in the pathogenesis of *K. pneumoniae*. Current studies are also increasingly focusing on the investigation of the capsule of *K. pneumoniae* and its regulation and expression as well as synthesis mechanisms (Chu et al., 2023; Muner et al., 2024; Sun et al., 2024; Tang et al., 2024). For *hvKp* isolates in particular, more and more genetic factors are being described that lead to attenuated capsule synthesis compared to other pathotypes and offer a survival advantage in the host organism (Walker et al., 2020). In particular, the presence of the transcriptional regulators RmpA and RmpA2 is associated with a hypermucoviscous phenotype and increased virulence (Lai et al., 2003; Cheng et al., 2010; Hsu et al., 2015; Lin et al., 2020; Chu et al., 2023).

Just recently, Russo et al. published a study where they investigated 49 *K. pneumoniae* strains possessing varying combinations of siderophore and other virulence genes associated with a hypervirulence phenotype and with different antibiotic resistance properties (Russo et al., 2024). Altogether 16 isolates were categorized as *hvKp* and 33 as *cKp* based on their behavior in a murine infection model. Biomarker presence, siderophore production, mucoviscosity, virulence plasmid homogeneities, and Kleborate virulence scores were measured and evaluated to accurately differentiate pathotypes. The presence of all the investigated biomarkers *iucA*, *iroB*, *peg-344*, *rmpA*, and *rmpA2* was most accurate (94%) to predict hypervirulence; whereas the presence of ≥ 4 of these biomarkers was most sensitive (100%). Even when using such an extensive and well-defined strain collection and informative models and logistic regressions, predictions of hypervirulence in *K. pneumoniae* isolates remains challenging.

In a recent paper, Masson et al., describe that *K. pneumoniae* O1 antigen prevents complement mediated killing when these isolates were compared to a larger collection of *K. pneumoniae* isolates with other O antigens (Masson et al., 2024). We did not see any link between pathotypes, presence of O antigens and growth and survival in serum samples or platelet concentrates (Figures 8, 9). Admittedly, our strain collection was less variable in terms of O-antigen structure. However, *K. pneumoniae* isolates with identical O1-antigens behaved differently in the two mentioned experimental settings suggesting the important role of components other than the O locus involved in growth and survival in blood or similar compartments.

Limitations of the study

Our study has several limitations. First, the classification into the three groups *cKp*, ESBL-*cKp* and *hvKp* is oversimplified because it ignores major differences in genome content, in addition to the group designations, between the isolates within a given group and across different categories. These differences may potentially also influence cell-biological behavior in the given experiments. Second,

we might have validated our experimental findings by including more than two isolates per given group. The limitation in isolate number per group is due to the fact that each experiment had been executed three times at least due to statistical validations, and that we included quite a high number of different adhesion, invasion and replication experiments and various cell line types. We have extensively tested and analyzed a larger set of cell lines and experimental settings beforehand, which is not described in greater details in this manuscript. Third, we are well aware that using isogenic strains would be beneficial for the experimental settings. For instance, using two strain types just differing by a *bla*_{ESBL}-containing plasmid (e.g., as a *cKp* vs. *ESBL-cKp*) would better specify some findings and let assume to point towards physiological and immunological behavior to a few genomic differences only. However, well-defined and characterized *K. pneumoniae* recipients were not available when we planned and started our experiments some years ago. Additionally, genetic strategies to cure plasmids safely from *K. pneumoniae* strains were not established. Forth, it would have been advantageous, if we have used a complete and entire strain set through all the experiments included. The selection and limitation to a smaller or slightly different isolate selection was due to organizational and infrastructural requirements and demands of QM/QC at the different research institutions involved.

Conclusions

In summary, the data from the *in vitro* infection experiments showed that *K. pneumoniae* isolates representing various pathotypes (*cKp*, *ESBL-cKp*, *hvKp*) differ in the host-pathogen interactions with regard to their invasion, resistance to phagocytosis and replication potential in macrophages. Thus, evidence was found that the successful spread of strains representing distinct *K. pneumoniae* lineages is not solely due to a survival advantage under selective pressure by antibiotics, which contributes to a better understanding of pathogenic mechanisms of *K. pneumoniae*. A stronger cytotoxic effect on intestinal cells was observed for *hvKp*, which presumably favors their translocation. Furthermore, it could be shown that the pure presence of siderophore genes have no positive influence on the proliferation of *K. pneumoniae* in blood.

Data availability statement

The data presented in the study are deposited in the ENA repository, project accession number PRJEB85663.

Author contributions

KK: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. YP: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision,

Validation, Writing – original draft, Writing – review & editing. LH: Formal analysis, Investigation, Methodology, Writing – review & editing. MP: Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. OK: Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. IB: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. AW: Investigation, Methodology, Validation, Visualization, Writing – review & editing. MF: Data curation, Investigation, Methodology, Validation, Writing – review & editing. HK: Conceptualization, Data curation, Resources, Writing – review & editing. SB: Data curation, Formal analysis, Investigation, Writing – review & editing. RG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. GW: Supervision, Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Resources.

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Conflict of interest

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

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Monte Carlo simulation to optimize polymyxin B dosing regimens for the treatment of Gram-negative bacteremia

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Objective: This study aimed to predict and evaluate the efficacy of various polymyxin B dosing regimens for Gram-negative bacteremia using Monte Carlo simulation, with a specific focus on assessing the efficacy in patients receiving continuous renal replacement therapy (CRRT). The goal was to optimize clinical dosing regimens and guide rational polymyxin B use in practice.

Methods: A total of 1,939 Gram-negative bacterial strains were analyzed, collected between April 2019 and December 2021 through the China Bloodstream Gram-negative Pathogens Antimicrobial Resistance and Virulence Surveillance Network (CARVIS-NET). Pharmacokinetic parameters of polymyxin B from existing literature were used to conduct a Monte Carlo simulation based on pharmacokinetic/pharmacodynamic (PK/PD) theory. The probability of target attainment (PTA) and cumulative fraction of response (CFR) were evaluated across various dosing regimens.

Results: The main pathogens of Gram-negative bacteremia were *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, all of which demonstrated high susceptibility to polymyxin B. For pathogens with a minimum inhibitory concentration (MIC) ≤ 1 mg/L, all regimens achieved PTA $>90\%$. However, when the MIC increased to 2 mg/L, the PTA for the 500,000 IU q12h regimen decreased to 77.53%, and at an MIC of 4 mg/L, none of the dosing regimens achieved a PTA $>90\%$. For *P. aeruginosa* and *K. pneumoniae* with MIC ≤ 0.5 mg/L, all regimens demonstrated effectiveness. However, at MIC ≥ 1 mg/L, significant declines in PTA were observed, with the 500,000 IU q12h and 1.25 mg/kg q12h regimens yielding suboptimal outcomes. In CRRT patients, PTA values declined further, particularly against *K. pneumoniae*, raising concerns about potential treatment failure.

Conclusion: Polymyxin B demonstrates high efficacy for Gram-negative bacteremia with MIC ≤ 1 mg/L. However, efficacy diminishes as MIC increases,

particularly for *P. aeruginosa* and *K. pneumoniae*, where 500,000 IU q12h and 1.25 mg/kg q12h regimens may result in suboptimal outcomes. For CRRT patients with *K. pneumoniae* bacteremia, therapeutic drug monitoring and dose adjustments are crucial to mitigate treatment failure risks.

KEYWORDS

polymyxin B, Monte Carlo simulation, Gram-negative bacteria, bacteremia, pharmacokinetics/pharmacodynamics (PK/PD)

1 Introduction

Polymyxins, a class of non-ribosomal cyclic cationic polypeptide antibiotics, were first isolated in 1947 by Japanese researchers from *Bacillus polymyxa* cultures. The group primarily includes five variants: A, B, C, D, and E, with polymyxin B and colistin (polymyxin E) being the most commonly used agents in clinical practice. Polymyxins exert their antibacterial effect by binding to lipopolysaccharides on the outer membrane of Gram-negative bacteria, leading to leakage of intracellular contents (Zavascki et al., 2007). Although initially approved for clinical use in the 1950s, concerns over nephrotoxicity and neurotoxicity, along with the emergence of more potent antibiotics, led to a decline in their use. However, in recent years, with the global rise in antibiotic resistance, particularly the emergence of carbapenem-resistant Gram-negative bacilli (CRGNB), positioning polymyxins as a last-line treatment for multidrug-resistant Gram-negative infections.

Compared to colistin, polymyxin B has more favorable pharmacokinetic properties and superior renal safety, making it a preferable choice for the treatment of invasive infections (Tsuji et al., 2019). However, significant variability exists in the recommended dosing regimens across countries and clinical guidelines. For instance, the Chinese product label recommends a fixed dose of 500,000 to 1,000,000 IU/day (Shanghai First Biochemical Pharmaceutical Co. L, 2020), while the FDA recommends a weight-based dosing of 15,000–25,000 IU/kg/day in divided doses (U.S. National Library of Medicine, 2020). International guidelines propose a weight-based regimen with a loading dose of 2.0 to 2.5 mg/kg (1mg=10,000 units) followed by a maintenance dose of 1.25 to 1.5 mg/kg every 12 hours (Tsuji et al., 2019). These discrepancies highlight the challenge of establishing an optimal dosing regimens for polymyxin B.

Previous PK/PD studies (Sandri et al., 2013; Nelson et al., 2015) have demonstrated a correlation between increased polymyxin B dosing and improved clinical outcomes. Suboptimal dosing, particularly with polymyxin B <15,000 units/kg/day, has been linked to insufficient serum drug concentrations and inadequate AUC/MIC ratios, often leading to poor outcomes and treatment failure in critically ill patients (Ismail et al., 2018; Xia and Jiang, 2021). Conversely, higher doses (≥ 200 mg/day) of polymyxin B have

been associated with reduced in-hospital mortality, but at the cost of increased nephrotoxicity. Several studies have confirmed that a daily dose of ≥ 200 mg/day is independently and significantly associated with a high risk of acute kidney injury (AKI) (Elias et al., 2010; Falagas et al., 2021). These findings underscore the need for individualized dosing strategies that account for both efficacy and safety.

Patients undergoing continuous renal replacement therapy (CRRT) represent a particularly complex population. Traditional practices suggest no need for polymyxin B dose adjustments in renal impairment or CRRT. However, recent studies indicate that CRRT increases the clearance of polymyxin B, potentially requiring higher doses to achieve therapeutic targets (Luo et al., 2022). This creates a challenge in selecting the appropriate dosing regimen for these patients.

Monte Carlo simulation analysis is a well-established approach for optimizing antibiotic dosing regimens. Previous studies, such as those by Sandri et al (Sandri et al., 2013), emphasized the importance of initiating therapy with a loading dose to achieve optimal drug exposure. Miglis et al (Miglis et al., 2018) further explored weight-based loading and fixed-dose regimens, demonstrating their potential in balancing efficacy and toxicity. Studies by Yu et al (Yu et al., 2021) and Wu et al. (2021) showed that adjusting polymyxin B doses in patients with renal insufficiency enhances the likelihood of achieving optimal drug exposure. However, studies on optimal dosing regimens for CRRT patients remain sparse, and the existing researches were primarily based on small, localized patient cohorts, often fail to incorporate bacterial resistance data or tailor treatment strategies for different pathogens (Luo et al., 2022; Wang et al., 2022b), which presents certain limitations.

Building on these findings, this study uses Monte Carlo simulation to evaluate different polymyxin B dosing regimens for treating Gram-negative bacteremia, including in CRRT patients, to provide clinical guidance on optimal dosing strategies. By integrating large-scale epidemiological data on bacterial resistance, our approach aims to optimize and personalize dosing strategies for critically ill patients with Gram-negative bacteremia caused by different pathogens, addressing a gap in the current literature and contributing to the development of more precise and effective treatment protocols.

2 Materials and methods

2.1 Bacterial strains

This study utilized a publicly available dataset from the CARVIS-NET comprising 1,939 Gram-negative bacterial isolates. These isolates were consecutively and non-repetitively collected from individual patients with clinically and laboratory-confirmed bloodstream infections across 21 centers in 20 cities in China between April 2019 and December 2021. All organisms were considered clinically significant based on local hospital criteria and isolated from high-quality specimens of each patient's first positive blood culture. The dataset includes community-associated and nosocomial bloodstream infections, with community-associated infections accounting for approximately 47.8%. Infection sources were classified based on clinical records into categories such as respiratory tract infections, urinary tract infections, alimentary tract infections, and others. Ethical approval for the initial data collection was obtained from the Human Research Ethics Committee of PUMCH (Ethics Approval Number: HS2755), as reported in the original study (Xi et al., 2022). No additional ethical approval was required for our analysis, as this study solely relies on publicly accessible data.

2.2 Antibiotic susceptibility and MIC determination

Antibiotic susceptibility testing for polymyxin B was performed using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI). The MICs were interpreted according to the CLSI M100-S32 guidelines or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. Specifically, MIC breakpoints for polymyxin B were as follows: For *Enterobacterales* and *Acinetobacter*, MICs ≤ 2 mg/L were considered susceptible, and MICs > 2 mg/L as resistant. For *Pseudomonas* MICs ≤ 4 mg/L were categorized as susceptible, and MICs > 4 mg/L as resistant (EUCAST, 2022). *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 were used as quality controls (Xi et al., 2022).

2.3 Polymyxin B PK/PD parameter targets

The PK/PD index for polymyxin B is the ratio of free drug area under the curve to MIC (f AUC/MIC) (Sandri et al., 2013; Tsuji et al., 2019). In this formula, AUC is calculated as Dose/CL where Dose represents the administered dose, CL refers to the drug clearance rate, and f denotes the fraction of unbound drug. The reference target value for this ratio is ≥ 10 , with a range of 3.5–28.0 (Hanafin et al., 2023). For specific pathogens, the target values are 20.8 for *P. aeruginosa*, 28.0 for *K. pneumoniae*, and 13.9 for *A. baumannii* (Yang et al., 2021).

2.4 Polymyxin B dosing regimens

Based on the polymyxin B product insert, relevant guidelines, and common clinical practice, the following intravenous dosing regimens were evaluated. All regimens were administered over at least 1 hour.

1. 500,000 IU q12h;
2. 1,000,000 IU q12h;
3. 1.25 mg/kg q12h;
4. Loading dose: 2 mg/kg over 1 hour, followed by maintenance: 1.25 mg/kg q12h;
5. Loading dose: 2.5 mg/kg over 1 hour, followed by maintenance: 1.5 mg/kg q12h.

2.5 Monte Carlo simulation of antimicrobial therapy

2.5.1 Polymyxin B PK parameters

Polymyxin B PK parameters were derived from population pharmacokinetic (PPK) studies in different patient populations. For patients with normal renal function, polymyxin B CL was reported as 0.028 ± 0.007 L/h/kg (Yu et al., 2022), based on adult patients with endogenous creatinine clearance ranging from 60 to 120 mL/min and bloodstream infections caused by carbapenem-resistant *K. pneumoniae*, as detailed in Table 1; while in patients undergoing CRRT, polymyxin B CL was slightly higher, measured at 0.033 ± 0.003 L/h/kg (Luo et al., 2022). The PPK analysis for CRRT patients was based on adult intensive care unit (ICU) patients with confirmed or suspected infections caused by carbapenem-resistant organisms. The mean fraction of unbound drug in plasma was 0.42 (range: 0.26–0.64) (Sandri et al., 2013; Zheng et al., 2023).

2.5.2 Monte Carlo simulation method

Monte Carlo simulation were performed using Oracle Crystal Ball (version 11.1.1.4.400) software to evaluate the efficacy of different polymyxin B dosing regimens. A total of 10,000

TABLE 1 Population pharmacokinetic parameters in different patient populations.

Demographic characteristics	Normal Renal Function	CRRT
Age (years)	68 (63–73)	65 (27–93)
Sex (male/female)	7/2	33/16
Weight (kg)	60 (55–65)	60 (41–91)
Creatinine clearance (ml/min)	89 (68–106)	–
CL (L/kg/h)	0.028 ± 0.007	0.033 ± 0.003

CRRT: continuous renal replacement therapy, CL: clearance rate

simulations were conducted, assuming that CL follows a normal distribution and f follows a uniform distribution, with a 95% confidence interval. Based on the PK/PD parameter targets, the probability of target attainment (PTA) was calculated for each dosing regimen at various MIC values. Custom MIC values and probability distributions were input into the simulation model, and corresponding PK/PD target values were adjusted. The cumulative fraction of response (CFR) was then calculated to express the expected probability of each dosing regimen achieving the target threshold against a population of pathogens. Generally, dosing regimens with PTA or CFR $\geq 90\%$ are considered appropriate for empirical antimicrobial therapy.

$$\text{CFR} = \sum_{i=1}^n \text{PTA}_i \times F_i$$

Among them, PTA_i refers to the probability of target attainment for a specific MIC value, and F_i represents the percentage of each MIC distribution within the population of bacterial strains.

3 Results

3.1 Distribution of MIC for Gram-negative bacteria

Among the 1,939 Gram-negative bacterial strains isolated, 1,724 (88.91%) belonged to the *Enterobacterales* family, while 207 (10.67%) were non-fermentative bacteria. The five most common pathogens identified were *E. coli* (896 strains, 46.21%), *K. pneumoniae* (612 strains, 31.56%), *P. aeruginosa* (95 strains, 4.90%), *A. baumannii* (82 strains, 4.23%), and *E. cloacae* (58 strains, 2.99%) (Table 2).

The susceptibility of these Gram-negative bacteria to polymyxin B varied across different infection sources, as shown in Table 3. Bloodstream infection isolates derived from catheter-related bloodstream infections exhibited the lowest susceptibility to polymyxin B (90.48%), whereas isolates from alimentary tract and cardiovascular system infections demonstrated 100% susceptibility.

The isolated Gram-negative bacteria demonstrated a high level of susceptibility to polymyxin B, with 94.07% of the isolates exhibiting an MIC ≤ 2 mg/L. *E. coli* showed the highest susceptibility, with MIC₅₀

and MIC₉₀ values of 0.25 mg/L and 0.5 mg/L, respectively. For *K. pneumoniae*, the MIC₅₀ and MIC₉₀ values were both 0.5 mg/L, while for *P. aeruginosa*, they were both 1 mg/L, and for *A. baumannii*, they were 0.5 mg/L and 1 mg/L, respectively (Table 4).

3.2 Efficacy of polymyxin B in treating Gram-negative bacteremia in patients with normal renal function

The PTA values of various polymyxin B dosing regimens for treating Gram-negative bacteremia are shown in Figure 1. As demonstrated in Figure 1A, all dosing regimens achieved PTA $\geq 90\%$ for Gram-negative bacteremia isolates with MIC ≤ 1 mg/L. However, when the MIC increased to 2 mg/L, 500,000 IU q12h showed a sharp decline in PTA to 77.53%, falling below the target threshold, and when the MIC increased to 4 mg/L, none of the regimens could achieve a PTA $> 90\%$.

For *P. aeruginosa* infections (Figure 1B), all dosing regimens achieved PTA $\geq 90\%$ at MIC ≤ 0.5 mg/L. However, when the MIC increased to 1 mg/L, 500,000 IU q12h failed to reach the effective target, with PTA dropping to 74.52%. Notably, when MIC increased to 2 mg/L, no regimen achieved PTA $> 90\%$.

For *K. pneumoniae* infections (Figure 1C), the simulation indicated that when the MIC ≤ 0.5 mg/L, all regimens reached the target. However, at an MIC of 1 mg/L, the 500,000 IU q12h and 1.25 mg/kg q12h regimens failed to reach the effective target. When the MIC increased to 2 mg/L, none of the regimens achieved a PTA $> 90\%$.

TABLE 3 Susceptibility of Gram-negative bacteria to polymyxin B across different infection sources.

Infection Sources	Number of isolates susceptible to polymyxin B	Total
Respiratory tract infection	313 (91.79%)	341
Urinary tract infection	254 (96.58%)	263
Alimentary tract Infection	51 (100%)	51
Central nervous system infection	15 (93.75%)	16
Liver abscess	80 (97.56%)	82
Biliary tract infection	187 (94.92%)	197
Abdominal infection of other organs (except liver and biliary tract infection)	147 (91.30%)	161
Pelvic infection (including puerperal infection)	16 (94.12%)	17
Skin and soft tissue infection	69 (98.57%)	70
Cardiovascular system infection	5 (100%)	5
Catheter-related bloodstream infections	38 (90.48%)	42
Others	649 (93.52%)	694
Total	1824 (94.07%)	1939

TABLE 2 Distribution of 1,939 Gram-negative bacteremia isolates.

Bacterial Species	Number of Isolates	Proportion (%)
<i>E. coli</i>	896	46.21%
<i>K. pneumoniae</i>	612	31.56%
<i>P. aeruginosa</i>	95	4.90%
<i>A. baumannii</i>	82	4.23%
<i>E. cloacae</i>	58	2.99%
Other Gram-negative bacteria	196	10.11%
Total	1939	100%

TABLE 4 MIC distribution of polymyxin B against Gram-negative bacteria (%).

Bacteria	MIC (mg/L)										MIC50 (mg/L)	MIC90 (mg/L)
	0.25	0.5	1	2	4	6	16	32	64	128		
<i>E. coli</i>	67.41	27.12	3.68	0.33	0.33	0.45	0.22	0	0	0.45	0.25	0.5
<i>K.pneumoniae</i>	33.82	57.68	4.25	1.31	0.33	0.82	0.98	0.33	0.16	0.33	0.5	0.5
<i>P. aeruginosa</i>	4.21	44.21	48.21	2.11	0	0	1.05	0	0	0	1	1
<i>A. baumannii</i>	35.37	52.44	8.54	0	1.22	0	0	1.22	0	1.22	0.5	1
<i>E. cloacae</i>	36.21	41.38	1.72	1.72	0	0	0	1.72	1.72	15.52	0.5	128
Total	46.98	39.50	6.24	1.34	0.62	0.62	0.57	0.26	0.31	3.56		

MIC50, Minimum inhibitory concentration required to inhibit the growth of 50% of isolates.
MIC90, Minimum inhibitory concentration required to inhibit the growth of 90% of isolates.

For *A. baumannii* infections (Figure 1D), the simulation showed that when the MIC ≤ 1 mg/L, all regimens reached the target. However, at an MIC of 2 mg/L, the 500,000 IU q12h and 1.25 mg/kg q12h regimens failed to reach the effective target. When the MIC increased to 4 mg/L, none of the regimens achieved a PTA > 90%.

3.3 Efficacy of polymyxin B in treating Gram-negative bacteremia in CRRT patients

Compared to patients with normal renal function, CRRT patients exhibited a decrease in PTA across all dosing regimens and target pathogens (Figure 2). As shown in Figure 2A, the PTA of 500,000 IU q12h regimen for all Gram-negative bacteria at MIC = 2 mg/L declined by approximately 13.6% (from 77.53% to 63.97%) in CRRT patients compared to those with normal renal function (Figure 1A). Specifically, for *P. aeruginosa* (Figure 2B), the PTA declined from 11.80% to 0.07%; for *K. pneumoniae* (Figure 2C), it dropped from 2.88% to 0.01%; and for *A.*

baumannii (Figure 2D), it declined from 45.26% to 24.21%. However, using a PTA ≥ 90% as the criterion for regimen selection, CRRT did not significantly impact treatment outcomes.

Considering the higher therapeutic demands for severe infections caused by multidrug-resistant Gram-negative bacteria, PTA was also evaluated with a more stringent ≥ 95% threshold for regimen selection (Paranos et al., 2022). Under this criterion, CRRT patients with *K. pneumoniae* infections (MIC ≥ 0.5 mg/L) could not achieve therapeutic efficacy with the 500,000 IU q12h regimen. Furthermore, neither the 1,000,000 IU q12h regimen nor the dosing strategy consisting of a loading dose of 2 mg/kg followed by a maintenance dose of 1.25 mg/kg q12h was effective when MIC ≥ 1 mg/L. Similarly, for *A. baumannii* infections, both the 1,000,000 IU q12h regimen and the aforementioned loading and maintenance dose strategy failed to achieve the effective target when MIC ≥ 2 mg/L.

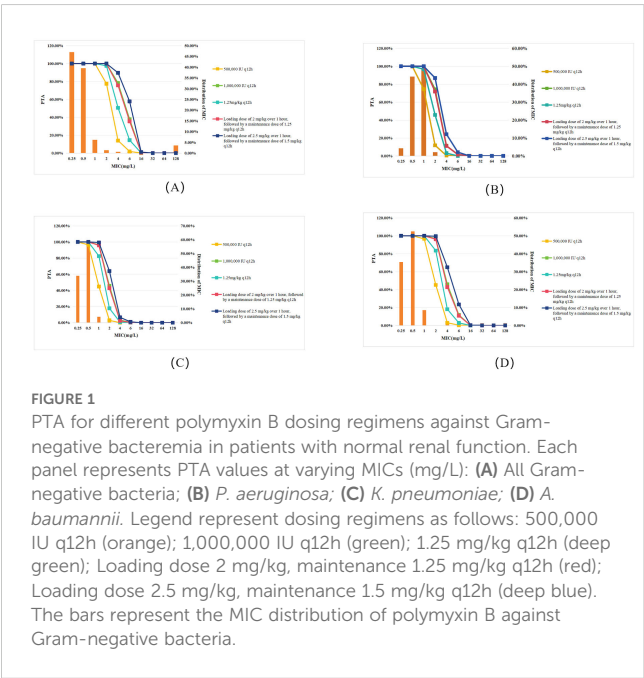
3.4 CFR values of polymyxin B dosing regimens for Gram-negative bacteremia

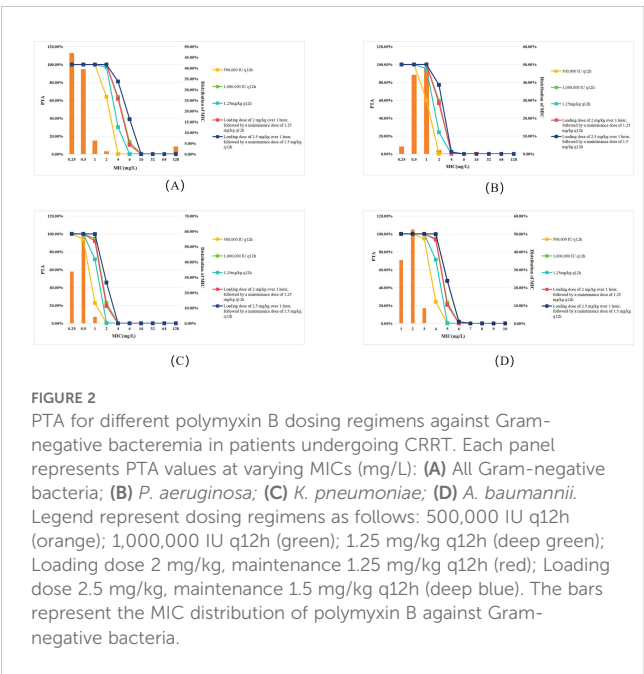
The CFR values for different polymyxin B dosing regimens in treating Gram-negative bacteremia in patients with normal renal function are shown in Table 5. The Monte Carlo simulation indicated that all simulated regimens achieved a CFR > 90% for Gram-negative bacteremia, suggesting good clinical efficacy. However, when the pathogen was *P. aeruginosa*, the 500,000 IU q12h regimen had a CFR value of 85.12%, indicating a potential for treatment failure.

In CRRT patients, all regimens showed a decrease in CFR compared to those with normal renal function (Table 6). However, using CFR ≥ 90% as the criterion for regimen selection, CRRT only affected the treatment of *K. pneumoniae*. For CRRT patients with *K. pneumoniae* infections, the CFR for the 500,000 IU q12h regimen dropped to 89.59%, indicating a potential risk of suboptimal treatment.

4 Discussion

With the widespread use of carbapenem antibiotics, CRGNB, which have emerged under the selective pressure of powerful





antibiotics, have become a major global public health threat. Compared to other infections, bloodstream infections caused by CRGNB have a significantly higher mortality rate. A study by [Falcone et al. \(2023\)](#) showed that the 30-day mortality rate for bloodstream infections caused by carbapenem-resistant bacteria (26.6%~43.2%) was significantly higher than that for infections caused by carbapenem-sensitive bacteria (13.7%).

Treatment options for CRGNB are extremely limited, with sensitive drugs including tigecycline, polymyxins, and ceftazidime-avibactam. Polymyxin B has high sensitivity against common Gram-negative bacteria. In this study, the sensitivity rate of polymyxin B against common Gram-negative bacteria ranged from 96.3% to 99.0%, consistent with another surveillance report showing sensitivity rates between 95.1% and 99.3% ([Liu and Ji, 2024](#)). Even for carbapenem-resistant strains, polymyxin B exhibited high efficacy, with sensitivity rates of 89.7%, 88.3%, 95.0%, and 99.0% for carbapenem-resistant *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* respectively. Despite its high sensitivity, several factors,

including the expression of antimicrobial activity, outdated drug labels, and uncertainties in susceptibility testing, have contributed to inconsistent clinical application of polymyxin B ([Tsuji et al., 2019](#)). Therefore, determining the appropriate dosing strategy is a key aspect of optimizing polymyxin B treatment regimens.

In our study, polymyxin B effectively treated Gram-negative bacteremia when the MIC was ≤ 1 mg/L. However, the effectiveness of the treatment regimen decreased when the infecting pathogens were *P. aeruginosa* or *K. pneumoniae*. At an MIC of 1 mg/L, the 500,000 IU q12h may result in suboptimal clinical outcomes. The CFR values further indicate that the 500,000 IU q12h carries a significant risk of treatment failure, especially in CRRT patients. This may be attributed to the lower sensitivity of *P. aeruginosa* to polymyxin B compared to other bacteria and the higher target values for *K. pneumoniae*. To optimize targeted therapy for these pathogens, higher off-label doses should be considered to ensure effective treatment. However, when the MIC increases to 2 mg/L, none of the simulated dosing regimens can achieve the PTA target ($\geq 90\%$) for bloodstream infections caused by *P. aeruginosa* or *K. pneumoniae*. According to [EUCAST \(2022\)](#) guidelines, the susceptibility breakpoint for polymyxins is 2 mg/L for *K. pneumoniae*, indicating that, in clinical practice, susceptibility testing may indicate sensitivity, but the clinical treatment outcomes might still be poor. It is important to note that the results of the Monte Carlo simulation are highly dependent on the input parameters and bacterial susceptibility. *f* AUC/MIC is considered the best PK/PD index for predicting the efficacy of polymyxin B, but there remains no consensus on the recommended target values, and the target values vary significantly across studies, leading to differences in study outcomes. For instance, [Wang et al. \(Wang et al., 2022a\)](#) proposed a target of 50 and concluded that in elderly patients with multidrug-resistant Gram-negative bacterial infections, doses of 50 mg and 75 mg q12h achieve an optimal balance between nephrotoxicity and efficacy. [Yu et al. \(2022\)](#) adopted bacteria-specific target values and found that for isolates with MIC ≤ 1 mg/L, a maintenance dose of 1 mg/kg q12h could achieve a PTA $>90\%$. In our study, we selected an *f* AUC/MIC target of 10, which is the average value required to reduce bacterial counts by 1 log₁₀ in a mouse thigh infection model for nine Gram-negative bacteria. For *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*, the

TABLE 5 CFR of polymyxin B dosing regimens for treating bacteremia in patients with normal renal function.

Pathogen Category	CFR(%)				
	500,000 IU q12h	1,000,000 IU q12h	1.25 mg/kg q12h	Loading dose 2 mg/kg, maintenance dose 1.25 mg/kg q12h	Loading dose 2.5 mg/kg, maintenance dose 1.5 mg/kg q12h
All Gram-negative bacteria	93.82	94.83	94.24	94.42	94.77
<i>P. aeruginosa</i>	85.12	98.28	96.36	98.37	98.78
<i>K. pneumoniae</i>	91.63	96.29	95.22	96.17	96.52
<i>A. baumannii</i>	96.27	96.84	96.46	97.13	97.16

CFR values $\geq 90\%$ (bold) are considered optimal for empirical therapy.

TABLE 6 CFR values of polymyxin B dosing regimens for treating bacteremia in CRRT patients.

Pathogen Category	CFR(%)				
	500,000 IU q12h	1,000,000 IU q12h	1.25 mg/kg q12h	Loading dose 2 mg/kg, maintenance dose 1.25 mg/kg q12h	Loading dose 2.5 mg/kg, maintenance dose 1.5 mg/kg q12h
All Gram-negative bacteria	93.58	94.52	94.04	94.40	94.59
<i>P. aeruginosa</i>	77.31	98.15	94.78	97.95	98.52
<i>K. pneumoniae</i>	89.59	95.69	94.43	95.55	96.48
<i>A. baumannii</i>	96.06	96.78	96.41	96.85	96.92

CFR values $\geq 90\%$ (bold) are considered optimal for empirical therapy.

selected targets were the doses required to reduce bacterial counts by 1 log₁₀, which were 28.0, 20.8, and 13.9, respectively (Landersdorfer et al., 2018; Society of Clinical Microbiology and Infection of China International Exchange and Promotion Association for Medical and Healthcare CMGotLMSotCMA and Clinical Microbiology Group of the Microbiology and Immunology Society of the Chinese Medical Association, 2020).

Critically ill patients often exhibit substantial pathophysiological changes, such as hepatic and renal dysfunction, hypoalbuminemia, extensive fluid resuscitation, and alterations in drug distribution volume, all of which can significantly impact the PK of antibiotics. In this study, the mean unbound fraction of polymyxin B was assumed to be 0.42, based on data derived from ICU patients. However, actual unbound fractions can vary considerably across individual patients, particularly in critically ill populations, potentially influencing the interpretation of therapeutic outcomes.

Polymyxin B is predominantly cleared through non-renal pathways, with less than 1% of the drug excreted unchanged in the urine (Zavascki et al., 2007). Consequently, dose adjustments are generally unnecessary in patients with renal insufficiency or those undergoing renal replacement therapy (RRT). However, CRRT, a vital intervention in critically ill patients, can significantly alter drug clearance, subsequently affecting PK/PD parameters and therapeutic outcomes. Evidence suggests that in CRRT patients, especially those receiving continuous venovenous hemodiafiltration (CVVHDF), polymyxin B clearance may increase (Luo et al., 2022; Hanafin et al., 2023; Pi et al., 2023). A study by Luo et al. (2022) showed that the clearance of polymyxin B in CRRT patients (1.95 L/h) was higher than in non-CRRT patients (1.5 L/h). Hanafin et al. (2023) reported that in CVVHDF patients, the steady-state AUC_{0-24h} was 50% lower than in patients not receiving CRRT, underscoring the need to consider the impact of CRRT on therapeutic outcomes. Consistent with these observations, our study demonstrated that CRRT patients exhibited a reduced PTA for polymyxin B across multiple dosing regimens compared to non-CRRT patients. When using PTA or CFR $\geq 90\%$ as the threshold for therapeutic effectiveness, CRRT had no significant impact on treatment outcomes except for *K. pneumoniae* infections. However, when applying the stricter criterion of PTA $\geq 95\%$, a notable shift in efficacy was observed. In CRRT patients, both the 1,000,000 IU q12h regimen and the dosing strategy of 2 mg/kg loading dose followed by a maintenance dose of 1.25 mg/kg q12h demonstrated reduced efficacy against *K. pneumoniae* and *A. baumannii*, particularly when the

MIC values ranged from 0.5 to 2 mg/L. Therefore, for these patients, it is recommended to enhance TDM and adjust drug dosing based on the monitoring results to ensure treatment efficacy and minimize the risk of resistance.

Due to the limited sample size of bloodstream infections caused by carbapenem-resistant bacteria, the bacterial susceptibility data used in this study are based on bloodstream infections caused by all Gram-negative bacteria. Since polymyxin B is primarily used for infections caused by carbapenem-resistant bacteria, differences in sensitivity to polymyxin B may lead to varying clinical outcomes. Data from the China Bacterial Resistant Investigation Collaborative System (BRICS) showed (Liu and Ji, 2024) that the resistance rate of *E. coli* to polymyxin B is 1.6%, while the rate increases to 10.3% in carbapenem-resistant *E. coli*. For *K. pneumoniae*, the resistance rate of to polymyxin B is 4.5%, rising to 11.7% in carbapenem-resistant strains. The resistance rate of *P. aeruginosa* is 0.7%, while the carbapenem-resistant strain has a slightly higher rate of 1%. Similarly, for *A. baumannii*, the resistance rate of to polymyxin B is 4.9%, increasing slightly to 5.0% in carbapenem-resistant strains. Susceptibility testing results showed that carbapenem resistance significantly affected the sensitivity of *E. coli* and *K. pneumoniae* to polymyxin B, but had a smaller impact on *P. aeruginosa* and *A. baumannii*. Therefore, a more conservative approach should be taken when interpreting CFR values for *E. coli* and *K. pneumoniae*.

The safety of polymyxin B is also an important consideration when optimizing dosing regimens. Nephrotoxicity is the main dose-limiting toxicity of polymyxin B, affecting up to 30% of patients (Phe et al., 2014; Rigatto et al., 2015; Oliota et al., 2019). A study by Elias et al., 2010 showed that a polymyxin B dose ≥ 200 mg/day was associated with a higher incidence of severe renal injury. Our study showed that as the drug dose increased, the probability of achieving the target concentration also increased, but higher doses were associated with an increased risk of severe renal injury. Weight-adjusted dosing regimens may result in suboptimal drug concentrations in underweight patients (Miglis et al., 2018; Hanafin et al., 2023), or significant adverse reactions in overweight patients (Wang et al., 2022a). Our study showed that when the MIC of the pathogen was ≤ 0.5 mg/L, a fixed-dose regimen of 500,000 IU q12h had similar efficacy to weight-adjusted dosing regimens, with potentially better safety. When the MIC increased to 1 mg/L, the 1,000,000 IU q12h regimen had similar efficacy to the loading-dose plus maintenance-dose regimen, but potentially better

safety. A fixed-dose regimen may be the better option after balancing efficacy and adverse reactions, but further studies are needed to determine the optimal regimen.

Although previous studies have explored the optimization of polymyxin B dosing regimens for the treatment of Gram-negative infections, these studies often focus on single-pathogen infections or lack considerations for the dynamic changes in critically ill patients, particularly those receiving CRRT. Furthermore, previous studies typically did not incorporate comprehensive bacterial resistance data or explore individualized treatment strategies for different pathogens. Our study addresses these gaps by combining large-scale epidemiological resistance data with Monte Carlo simulations to optimize polymyxin B dosing regimens for various Gram-negative pathogens, including those in CRRT patients. However, this study has several limitations that should be acknowledged. First, all polymyxin B dosing regimens in the simulation were based on monotherapy, without accounting for the potential synergistic effects or improved clinical outcomes associated with combination therapy. The rapid spread of plasmid-mediated MCR-1 resistance genes has significantly impacted the effectiveness of polymyxin antibiotics (Liu et al., 2016). Combination antibiotic therapy presents an attractive option for treatment. Additionally, factors such as the safety of high-dose polymyxin B administration, the presence of heteroresistance, and the link between colistin resistance and increased in-hospital mortality further highlight the advantages of combination therapy (Tsuji et al., 2019). However, our study did not explore the role or impact of combination regimens, which may limit the applicability of the findings in clinical practice. Additionally, the population pharmacokinetic data used in this study were derived from a relatively small cohort, necessitating further data collection and validation to improve model accuracy. Finally, it is important to note that critically ill patients often exhibit significant variability in PK/PD parameters due to dynamic physiological changes, which should be carefully considered when applying these results to clinical practice.

5 Conclusion

Our study showed that polymyxin B demonstrated good clinical efficacy for Gram-negative bacteremia, especially in pathogens with lower MICs. However, for infections caused by *P. aeruginosa* and *K. pneumoniae* with higher MIC values, lower-dose regimens, such as 500,000 IU q12h, may result in suboptimal treatment outcomes. In patients undergoing CRRT, both PTA and CFR values decrease across all dosing regimens compared to those with normal renal function, with a notable reduction in efficacy against *K. pneumoniae*. These findings highlight the importance of adjusting dosing regimens based on renal function and MIC values to optimize clinical outcomes. We recommend the routine measurement of MICs and individualized therapy to ensure effective treatment. Furthermore, integrating PK/PD modeling with local resistance patterns and TDM can assist clinicians in selecting the most appropriate antibiotic regimens for Gram-negative bacteremia, especially in complex patient populations such as those receiving CRRT.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

ZH: Validation, Writing – original draft. YY: Conceptualization, Validation, Writing – original draft. CW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Characterization of carbapenem-resistant *Klebsiella pneumoniae* in bloodstream infections: antibiotic resistance, virulence, and treatment strategies

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Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections pose a major clinical challenge due to multidrug resistance. This study evaluated the clinical features, antibiotic resistance mechanisms, virulence factors, and the potential therapeutic impact of berberine hydrochloride (a traditional Chinese medicine) in CRKP infections.

Methods: Ninety-four CRKP isolates from bloodstream infections at the First Affiliated Hospital of Zhejiang Chinese Medical University were characterized for carbapenemase genes, antibiotic susceptibility, and virulence determinants. Clinical data were analyzed to identify risk factors for CRKP infection, and the *in vitro* antibacterial activity of berberine hydrochloride was assessed.

Results: Most of the isolates (71.3%) were from the intensive care unit (ICU) patients. The *bla*_{KPC} gene was the predominant resistance mechanism (62.77%), while the virulence genes *uge* (93.62%) and *wabG* (92.55%) were highly prevalent. ICU admission, male sex, respiratory diseases, invasive procedures, prior use of third-generation cephalosporinase inhibitors, and absence of traditional Chinese medicine treatment were linked to poorer outcomes. Importantly, berberine hydrochloride inhibited CRKP growth *in vitro*, with a minimum inhibitory concentration (MIC) of 125 mg/mL.

Conclusion: Our study reveals the multifaceted resistance and virulence profiles of CRKP in bloodstream infections and highlights the potential clinical value of berberine hydrochloride as an adjunctive therapeutic agent. These findings support further clinical investigations into incorporating traditional Chinese medicine to improve outcomes in patients with CRKP bloodstream infections.

KEYWORDS

CRKP, bloodstream infections, clinical features, multidrug resistance, risk factors, berberine hydrochloride

Introduction

Klebsiella pneumoniae (KP) is a significant gram-negative opportunistic pathogen that colonizes human skin, the respiratory tract, and the intestine. A weakened defense response or compromised natural barriers can lead to various infections, such as those of the urinary tract and bloodstream, pneumonia, and liver abscesses (Jondle et al., 2018; Aghamohammad et al., 2020). Data from the China Antimicrobial Surveillance System from 2014–2019 underscore the prevalence of KP, which constitutes 10.2% of the bacteria isolated from blood samples (China Antimicrobial Resistance Surveillance System, 2021). Moreover, the resistance rates to imipenem and meropenem range from 6.6% to 11.7%, showing an increasing trend over the years (China Antimicrobial Resistance Surveillance System, 2021), thereby posing a significant challenge to their clinical treatment.

In recent years, the widespread use of broad-spectrum antibiotics has led to the emergence of highly drug-resistant and pathogenic strains of KP. The emergence of carbapenem-resistant Enterobacteriaceae (CRE) represents a critical challenge in clinical anti-infection therapy (Barnes et al., 2019). CRE encompasses a spectrum of resistance mechanisms, including β -lactamase production, the modification of porins, and changes in bacterial cell membrane permeability (Anderson and Boerlin, 2020). These mechanisms confer resistance against conventional antibiotics, thus complicating treatment strategies. Enterobacter species can produce carbapenemase and extended-spectrum β -lactamase (ESBL) enzymes, which confer resistance to third-generation antibiotics, thereby further increasing the therapeutic challenge. Ambler's molecular classification divides carbapenemases into three groups: class A (carbapenemases, such as KPC and GES), class B (metallo β -carbapenemases or MBLs, such as VIM, IMP, and NDM), and class D (oxacillinase or OXA, such as OXA-48) (Bush et al., 1995; Queenan and Bush, 2007; Bush and Jacoby, 2010). Additionally, the emergence of hypervirulent KP (hvKP) strains has increased mortality rates through more challenging treatments (Shon et al., 2013; Dai and Hu, 2022; Han et al., 2022; Yang et al., 2022). Among these strains, virulence genes such as *uge* and *wabG* are commonly found, contributing to their high pathogenicity (Remya et al., 2019; Wang et al., 2020; Kot et al., 2023). The high pathogenicity of these strains is attributed to virulence genes such as *uge* and *wabG*, which along with other factors (such as capsular polysaccharides and biofilm formation), play crucial roles in the virulence of KP (Srinivasan et al., 2012; Dunstan et al., 2023).

Rising infections and the emergence of CRKP strains have intensified the clinical challenge of managing multidrug-resistant pathogens. Previous studies integrating traditional Chinese and Western medicine have demonstrated that compounds such as scutellarin can block biofilm formation (Yin et al., 2016) and that glucoside B can inhibit bacterial efflux pumping activity (Zhong Haiqin and Ting, 2013), thereby increasing antimicrobial efficacy. Despite these promising findings, the therapeutic potential of berberine hydrochloride (a well-established traditional Chinese medicine) remains underexplored in the context of CRKP infections. Given the limited effectiveness of conventional antibiotics against CRKP, we hypothesize that berberine hydrochloride can mitigate key resistance mechanisms and

improve antibacterial outcomes. To test this hypothesis, our study aimed to evaluate the *in vitro* efficacy of berberine hydrochloride against CRKP isolates and elucidate its role as an adjunct treatment strategy, ultimately bridging the gap between carbapenem resistance challenges and innovative, integrative therapeutic approaches.

In this study, we comprehensively evaluated the clinical features, antibiotic resistance profiles, virulence-associated molecular characteristics, and risk factors for CRKP bloodstream infections. In parallel, we assessed the *in vitro* efficacy of berberine hydrochloride as an adjunctive therapeutic strategy. These findings not only provide a robust theoretical foundation for the prevention, control, and clinical management of CRKP infections but also underscore the critical role of traditional Chinese medicine in enhancing patient outcomes.

Materials and methods

Data collection

A total of 94 nonrepetitive clinical CRKP strains were isolated from the blood samples of 94 patients (60 males and 34 females) at the First Affiliated Hospital of Zhejiang Chinese Medical University from June 2017 to October 2022. The inclusion criteria were as follows: (1) the isolate was confirmed as *Klebsiella pneumoniae* with resistance to carbapenems according to established CLSI guidelines, (2) the isolate was obtained from a patient diagnosed with a clinically significant bloodstream infection, and (3) complete clinical and microbiological data were available for analysis. The exclusion criteria included (1) duplicate strains from the same patient, defined as multiple isolates with identical phenotypic and genotypic profiles during the same hospitalization period; (2) isolates that did not meet the laboratory criteria for CRKP on the basis of their resistance profiles; and (3) isolates with incomplete or missing clinical information. Patient data, including sex, age, duration of hospitalization, underlying diseases, antibiotic treatment, traditional Chinese medicine treatment, history of invasive procedures, and patient prognosis, were collected to investigate the risk factors associated with CRKP bloodstream infections.

Antibiotic susceptibility testing

All CRKP isolates were identified via matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany). Antibiotic susceptibility testing of the 94 clinical CRKP isolates was conducted via the Vitek-2 Compact system (Biomérieux, Marcy l'Etoile, Lyon, France). The tested antibiotics included amoxicillin, ampicillin, ceftazidime, cefepime, cefoperazone, ceftriaxone, ertapenem, imipenem, meropenem, piperacillin, ciprofloxacin, levofloxacin, amikacin, gentamycin, tobramycin, polymyxin, tigecycline, nitrofurantoin, aztreonam and puromycin. The minimum inhibitory concentrations (MICs) of meropenem, imipenem, and ertapenem were determined via the broth

microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) 2020-M100 guidelines. Quality control strains, including *Klebsiella pneumoniae* (ATCC700603), *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* (ATCC25913), and *Escherichia coli* (ATCC25922), were obtained from the China National Health Inspection Center.

Carbapenem resistance detection and virulence phenotyping of CRKP isolates

The carbapenem-resistant phenotype was confirmed via carbapenem inhibitor enhancement experiments conducted via the disc diffusion method (Hua et al., 2020). Briefly, a single colony of the CRKP isolate was diluted to a bacterial suspension (0.5 McFarland standard) using sterile saline, followed by spreading onto Mueller Hinton (MH) agar plates. Four discs of carbapenem (either imipenem or meropenem) were placed on the agar surface. One disc served as a control, while the second, third, and fourth discs were treated with 10 μ L of 50 mg/ml APB (3-aminophenyl boronic acid hydrochloride), 10 μ L of 0.5 M EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate), and 10 μ L of 50 mg/ml APB mixed with 10 μ L of 0.5 M EDTA solution, respectively. The plates were then incubated overnight at 35°C, after which the diameter of the antibacterial zone around the paper discs was measured. The results were interpreted according to established guidelines.

Next, the mucous phenotype was confirmed via a wire drawing experiment. A single colony from the agar plate was picked via a sterile inoculation ring. The mucus filament length exceeding 5 mm in three independent replicates represented a high mucous phenotype. Furthermore, serum collected from healthy individuals was used for the serum resistance test. The bacterial suspension from the overnight culture was diluted with Luria-Bertani (LB) broth (0.5 McFarland standard), mixed with serum, and incubated at 35°C for 0, 1, 2, and 3 h. After tenfold dilution, the mixture was spread onto MH agar plates and incubated overnight. Serum resistance was evaluated on the basis of colony-forming unit (CFU) counts (Chen and Kreiswirth, 2018; Mitra et al., 2019).

Biofilm formation experiments were conducted via the crystal violet staining method (Naparstek et al., 2014). The diluted bacterial suspension was added to a 96-well plate, which was subsequently sealed and incubated overnight at 35°C. After washing three times with neutral phosphate-buffered saline (PBS), 1% crystal violet was added for 15 min. After repeatedly rinsing with water and drying, anhydrous ethanol was added to dissolve the crystal violet, and the optical density (OD) was measured at 590 nm. The OD values indicate the extent of adhesion of the strain biofilm to the contact surface of the 96-well polystyrene plate.

Detection of carbapenem resistance genes and virulence-associated genes

Genomic DNA isolation from CRKP isolates was performed via the thermal lysis method, followed by the detection of genes

conferring carbapenem resistance and virulence via polymerase chain reaction (PCR). The primers used were designed according to previous studies (Candan and Aksöz, 2015; Makharita et al., 2020). The detailed primer sequences and the PCR parameters used for this study are listed in [Supplementary Tables S1–S4](#). The positive PCR products were subsequently sequenced (Shanghai Sangon Biotech Co., Ltd.), and the results were analyzed via the BLAST tool available at NCBI.

In vitro minimum inhibitory concentration (MIC) assay

Berberine hydrochloride was serially diluted in MH broth to final concentrations of 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 mg/mL. These concentration ranges were selected on the basis of preliminary experiments and literature reports indicating effective inhibitory levels against multidrug-resistant bacteria. CRKP strains were cultured in MH broth and exposed to the various concentrations of berberine hydrochloride, whereas the control groups received an equal volume of MH broth. The cultures were incubated at 37°C for 24 h, with bacterial growth was monitored by measuring the OD at 490 nm every 2 h. After the 24-hours incubation period, aliquots from each culture were spread onto MH agar plates and further incubated for 24 h to assess colony formation. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of berberine hydrochloride that completely inhibited bacterial growth, as evidenced by the absence of colonies on the agar plates.

Statistical analysis

All the figures and graphs were prepared via GraphPad Prism 9.5 software. The antibiotic susceptibility profiles of the CRKP strains were analyzed via WHONET 5.6, and all the statistical analyses were performed with SPSS 25.0. For univariate analysis, the χ^2 test and *t*-test were employed for qualitative and quantitative data, respectively, with a *P*-value of < 0.05 considered statistically significant. Variables meeting this threshold were then included in a forward binary logistic regression model to calculate the odds ratios (ORs) of related risk factors. Additionally, comparisons among multiple groups were carried out via one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

Results

Department distribution of clinical CRKP isolate data

All the nonrepetitive clinical CRKP strains (94 in total) were isolated from blood samples collected from patients admitted to 12 different departments, including the ICU (71.28%, *n* = 67), hematology (12.77%, *n* = 12), medical oncology (4.26%, *n* = 4),

gastroenterology (3.19%, $n = 3$), and other departments (8.48%, $n = 8$) (Figure 1).

Antibiotic susceptibility profiles

All the CRKP isolates were identified as multidrug-resistant strains, posing a major challenge for both treatment and infection control in healthcare settings. The antibiotic susceptibility of these strains was evaluated against 20 commonly used clinical antibiotics. The results revealed that the isolates exhibited 100% resistance to piperacillin, meropenem, ertapenem, ampicillin, ceftriaxone, and amoxicillin (Table 1). The resistance rates ranged from 75% to 99% for cefoperazone, ceftazidime, cefepime, aztreonam, tobramycin, ciprofloxacin, levofloxacin, imipenem, nitrofurantoin, and gentamycin (Table 1). In contrast, lower resistance rates (<75%) were observed for puromycin, polymyxin, tigecycline, and amikacin (Table 1). Notably, CRKP strains presented high sensitivity to polymyxin, with a resistance rate of only 5.32% (Table 1), underscoring its potential as a last-resort therapeutic agent. Furthermore, the high resistance levels, particularly in high-risk hospital areas such as the ICU, suggest that these isolates may be spreading clonally within the hospital environment.

Carbapenem-resistant genotypes and phenotype analysis of CRKP strains

Our sequencing results confirmed the genotypic distribution of carbapenem resistance among the CRKP isolates. The *bla*_{KPC} was the most prevalent and was detected in 59 strains (62.77%), followed by the *bla*_{NDM} gene in 14 strains (14.89%) (Table 2).

Additionally, minor carbapenemase genes, including *bla*_{VEB}, *bla*_{OXA-48}, *bla*_{GES}, and *bla*_{IMP}, were identified, while the *bla*_{VIM} gene was notably absent (Table 2), suggesting the presence of alternative resistance mechanisms.

Phenotypic analysis of carbapenemase activity further revealed that 77.6% of the strains produced class A serine proteases, 10.6% produced class B metalloenzymes, and 11.7% coproduced both types of carbapenemases (Table 2). These findings underscore the genetic diversity of CRKP strains and their potential for widespread dissemination in healthcare settings. The predominance of *bla*_{KPC}, in particular, suggests a high risk of hospital transmission due to its association with clonal expansion in nosocomial outbreaks.

Virulence-associated genotypes and phenotype analysis of CRKP strains

We next analyzed the distribution of virulence genes among the CRKP isolates to elucidate their potential impact on hospital transmission and clinical outcomes. The results revealed that the virulence genes *uge* and *wabG* were the most prevalent, with positive rates of 93.62% and 92.55%, respectively (Table 2). These genes, known to be involved in capsule biosynthesis and immune evasion, may facilitate persistent colonization in hospital environments. Other virulence determinants were detected at lower frequencies: *fimH* (65.96%), *rmpA* (44.68%), *iroN* (31.91%), *aerobactin* (9.57%), *alls* (2.13%), *mrkD* (2.13%), and *wcaG* (1.06%) (Table 2). Notably, *iutA*, *entB*, and *magA* were absent from our isolates (Table 2).

In addition to genotypic profiling, we also assessed the phenotypic virulence characteristics of the CRKP strains. Only 15 strains (15.79%) presented a hypermucoviscous phenotype, whereas

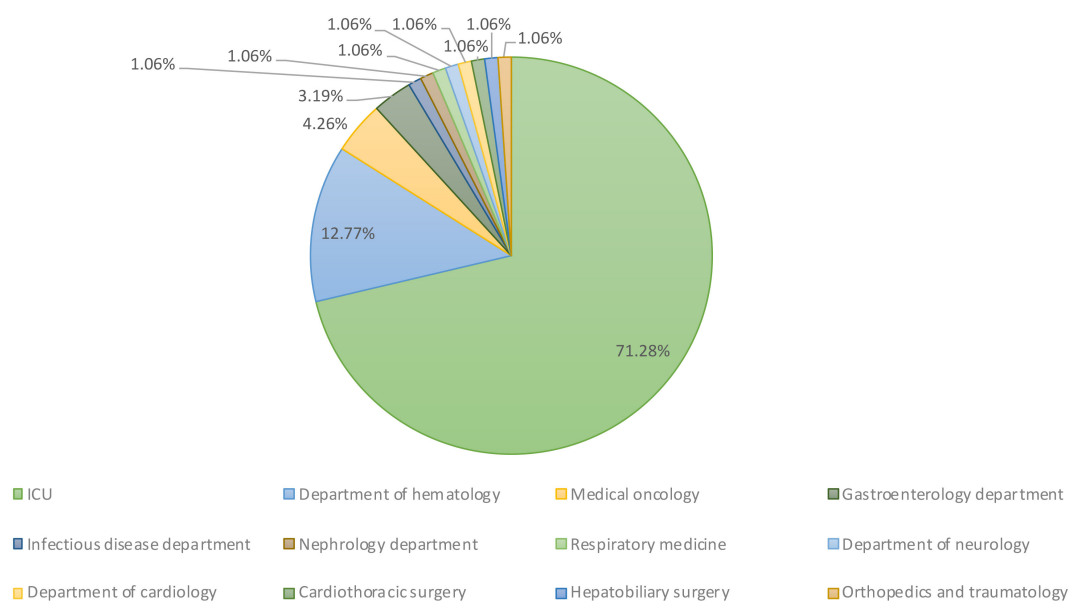


FIGURE 1

Department distribution of 94 clinical CRKP bloodstream infection strains. These strains were collected from a total of 12 different departments within the hospital setting.

TABLE 1 Antibiotic susceptibility profiles of 94 clinical CRKP strains.

Antibiotics	No.	%R	No.	%I	No.	%S
β-lactams						
Amoxicillin	94	100	0	0	0	0
Ampicillin	94	100	0	0	0	0
Ceftazidime	91	96.81	1	1.06	2	2.13
Cefepime	82	87.23	2	2.30	10	10.64
Cefoperazone	93	98.94	0	0	1	1.06
Ceftriaxone	94	100	0	0	0	0
Ertapenem	94	100	0	0	0	0
Imipenem	92	97.87	0	0	2	2.13
Meropenem	94	100	0	0	0	0
Piperacillin	94	100	0	0	0	0
Aztreonam	90	95.74	1	1.06	3	3.19
Quinolones						
Ciprofloxacin	84	89.36	10	10.64	0	0
Levofloxacin	83	88.30	6	6.38	5	5.32
Aminoglycosides						
Amikacin	69	73.40	24	25.53	1	1.06
Gentamycin	79	84.04	15	15.96	0	0
Tobramycin	73	77.66	16	17.02	5	5.32
Polypeptide						
Polymyxin	5	5.32	89	94.68	0	0
Tetracyclines						
Tigecycline	49	52.13	39	41.49	6	6.38
Nitrofurans						
Nitrofurantoin	80	85.11	1	1.06	13	13.83
Others						
Puromycin	63	67.02	30	31.91	1	1.06

R, resistant; I, intermediate; S, susceptible.

the remaining strains presented a normal phenotype (Table 3). The capsular serotype gene was detected in a mere 3 strains (3.19%), with the K1 and K2 serotypes found in two and one strains, respectively; the other major serotypes (K1, K2, K5, K20, K54, and K57) were not identified in the remaining isolates (Table 3). Furthermore, serum resistance and biofilm formation assays provided additional insights into the potential for nosocomial dissemination. Among the 94 strains, 50 (53.9%) were serum resistant, 32 (34.04%) were moderately sensitive, and 12 (12.77%) were highly sensitive (Table 3). In terms of biofilm formation, 6 strains (6.38%) presented strong biofilm-forming ability, 35 strains (37.23%) presented intermediate ability, and 53 strains (56.38%) presented weak biofilm formation (Table 3). Taken together, the high prevalence of virulence genes such as *wabG*, *fimH*, *rmpA*, and

iroN among CRKP bloodstream isolates highlights their potential role in facilitating persistent colonization and transmission within hospital settings. Although only a minority of isolates exhibited high virulence-associated phenotypes (e.g., hypermucoviscosity and capsular serotypes K1/K2), the presence of these factors may contribute to severe infections and nosocomial outbreaks.

Risk factors and multivariate analysis of CRKP bloodstream infections

The results revealed statistically significant differences in several factors associated with poor prognosis in patients with CRKP bloodstream infections. These factors included admission to the ICU ($P < 0.01$), male sex ($P = 0.022$), absence of traditional Chinese medicine treatment ($P = 0.09$), presence of respiratory disease ($P = 0.010$), history of invasive procedures ($P < 0.001$), and treatment with third-generation cephalosporin enzyme inhibitors ($P = 0.005$) (Table 4). These findings suggest that these variables could serve as risk factors for poor prognosis in patients with CRKP bloodstream infections. For invasive procedures, four specific factors, namely, venous intubation, nasogastric tube placement, hemodialysis, and arterial catheterization, were identified as significant risk factors for poor prognosis in patients with CRKP bloodstream infections ($P < 0.05$) (Table 4).

Furthermore, the results of multivariate binary logistic regression analysis demonstrated that treatment with traditional Chinese medicine had a significant positive effect on poor prognosis (OR, 4.025; 95% confidence interval (CI), 1.018-15.98), indicating a beneficial effect on patient outcomes (Table 5). Conversely, respiratory diseases (OR, 0.099; 95% CI, 0.014-0.701), venous intubation (OR, 0.058; 95% CI, 0.016-0.214), and treatment with third-generation cephalosporin enzyme inhibitors (OR, 0.042; 95% CI, 0.050-0.945) had a significant negative impact on prognosis (Table 5). Overall, traditional Chinese medicine, along with specific patient characteristics and treatments, serves as a crucial independent risk factor affecting the prognosis of CRKP infections. These findings align with those of previous studies and underscore the importance of these factors in the clinical management and treatment of CRKP bloodstream infections.

Berberine hydrochloride inhibited CRKP growth *in vitro*

Previous studies have demonstrated that berberine hydrochloride exhibits antimicrobial activity against various resistant bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Cutibacterium acnes* (Chu et al., 2016; Sun et al., 2023; Zhou et al., 2023; Liu et al., 2024). In our *in vitro* experiments, we determined the minimum inhibitory concentration (MIC) of berberine hydrochloride against CRKP strains to be 125 mg/mL (Figure 2). At concentrations of 125 mg/mL and above, berberine hydrochloride significantly inhibited CRKP growth, leading to complete bacterial elimination (Figure 2). After the broth was

TABLE 2 Detection of carbapenemase genes and virulence genes in 94 CRKP strains.

Genotypes	No.	%
Carbapenemase genes		
<i>bla</i> _{KPC}	59	62.77
<i>bla</i> _{NDM}	14	14.89
<i>bla</i> _{VEB}	5	5.32
<i>bla</i> _{OXA-48}	2	2.13
<i>bla</i> _{GES}	2	2.13
<i>bla</i> _{IMP}	1	1.06
<i>bla</i> _{VIM}	0	0
Virulence genes		
<i>uge</i>	88	93.62
<i>wabG</i>	87	92.55
<i>fimH</i>	62	65.96
<i>rmpA</i>	42	44.68
<i>iroN</i>	30	31.91
<i>aerobactin</i>	9	9.57
<i>alls</i>	2	2.13
<i>mrkD</i>	2	2.13
<i>wcaG</i>	1	1.06
<i>iutA</i>	0	0
<i>entB</i>	0	0
<i>magA</i>	0	0

transferred to agar plates and incubated for 24 h, no bacterial growth was observed, confirming the bactericidal effect. These findings suggest that berberine hydrochloride has potent inhibitory effects on CRKP *in vitro*, supporting its potential as a therapeutic agent against these resistant strains.

Discussion

Klebsiella pneumoniae (KP), the second most prevalent Gram-negative pathogen in China, poses escalating threats due to rising carbapenem resistance (CRKP), particularly in bloodstream infections and immunocompromised populations. CRKP-associated blood stream infections often correlate with high mortality, prolonged hospitalization, and therapeutic failure, necessitating sustained surveillance in high-risk settings like ICUs (Zhang et al., 2018). Unlike our previous multisource CRKP analysis (Zhu et al., 2022), this study specifically characterizes 94 bloodstream infections derived CRKP strains, revealing near-universal multidrug resistance linked to nosocomial clustering and antibiotic overuse. Notably, polymyxin retained efficacy (5.32% resistance rate), though emerging resistance underscores the urgency for judicious use.

TABLE 3 Phenotype detection of 94 clinical CRKP strains.

Phenotypes	No.	%
Carbapenem-resistant		
class A	73	77.66
class B	10	10.64
class A and B	11	11.7
Mucous phenotype		
high mucous	15	15.79
normal	79	84.04
Serum resistant		
serum resistant	50	53.19
intermediate sensitive	32	34.04
highly sensitive	12	12.77
Capsular serotype		
K1	2	2.13
K2	1	1.06
K5	0	0
K20	0	0
K54	0	0
K57	0	0
Biofilm formation capacity		
strong	6	6.38
intermediate	35	37.23
weak	53	56.38

The carbapenem-resistant phenotypes of CRKP strains can be categorized into three classes: Class A indicates the production of class A serine proteases; Class B indicates the production of class B metalloenzymes; and Class A and B indicate the production of both class A and class B enzymes.

These findings mandate three clinical priorities: (1) enhanced bloodstream infections focused CRKP surveillance to track resistance evolution in ICUs and transplant units; (2) restricted polymyxin deployment guided by rapid susceptibility testing to preserve its efficacy; and (3) implementation of antibiotic stewardship programs targeting carbapenem and cephalosporin overuse to mitigate multidrug resistance amplification. Future research should prioritize combinatorial therapies leveraging polymyxin’s residual activity while exploring alternative agents against resistant subpopulations.

Our findings demonstrate that carbapenemase production [predominantly *bla*_{KPC} (62.70%) and *bla*_{NDM} (14.89%)] remains the primary resistance mechanism in CRKP bloodstream infections, contrasting with global reports highlighting *bla*_{CTX-M} prevalence in extended-spectrum beta-lactamase (ESBL)-mediated resistance (Scheuerman et al., 2018). Notably, the convergence of antimicrobial resistance and virulence markers presents critical clinical challenges. The high *fimH* gene prevalence (67.7%) in KPC-positive strains suggests enhanced mucosal adhesion and

TABLE 4 Clinical features of 94 CRKP bloodstream infection patients.

Variables	Total n= 94 (%)	Poor prognosis		P
		Yes, n= 66 (%)	No, n= 28 (%)	
General information				
Gender: male	60 (63.83%)	47 (71.21%)	13 (46.43%)	0.022*
Age: ≥ 60	72 (76.60%)	54 (81.82%)	18 (64.28%)	0.066
ICU	65 (69.15%)	53 (80.30%)	12 (42.86%)	0.000**
Concomitant disease				
Hypertension	53 (56.38%)	36 (54.54%)	17 (60.71%)	0.457
Respiratory diseases	27 (28.72%)	25 (37.88%)	2 (7.14%)	0.010*
Heart failure	1 (1.06%)	1 (1.52%)	0	1.000 ^b
Heart disease	18 (19.15%)	12 (18.18%)	6 (21.43%)	0.714
Hematological-malignancies	14 (14.89%)	10 (15.15%)	4 (14.28%)	1.000 ^a
Invasive procedure				
Venous cannula	48 (51.06%)	43 (65.15%)	5 (17.86%)	0.000**
Nasogastric tube	36 (38.30%)	30 (45.45%)	6 (21.43%)	0.028*
Hemodialysis	16 (17.02%)	16 (24.24%)	0	0.002** ^b
Artery place pipe	15 (15.96%)	15 (22.73%)	0	0.004** ^b
Traditional Chinese medicine treatment	29 (30.85%)	15 (22.73%)	14 (50.00%)	0.009**
Third generation cephalosporin enzyme inhibitor treatment	9 (9.57%)	5 (7.58%)	4 (14.28%)	0.005**

^aChi-square test continuity correction; ^bFisher exact concept method; bold text indicates ***P* < 0.01; **P* < 0.05.

immune evasion capabilities, while capsule serotype K1/K2 and hypermucoviscous phenotypes (15.96%) in our cohort align with established hypervirulence markers (*alls*, *uge*, *wabG* genes). Paradoxically, limited biofilm formation (6.38%) and serum sensitivity (12.77%) reflect the evolutionary trade-off between virulence and resistance mechanisms (Schroeder et al., 2017; Cepas and Soto, 2020). These findings highlight the need for routine screening of *bla*_{KPC} and *bla*_{NDM} carbapenemases alongside

virulence markers (e.g., *fimH*, capsule typing) to guide combination therapies. Enhanced infection control protocols targeting hv-CRKP transmission, especially for K1/K2 serotypes, are also crucial. Additionally, developing rapid diagnostics that integrate resistance genotyping and virulence profiling is essential. Future research should focus on longitudinal surveillance of hv-CRKP evolutionary and clinical validation of virulence-inhibiting adjuvants to complement existing carbapenemase-targeted therapies.

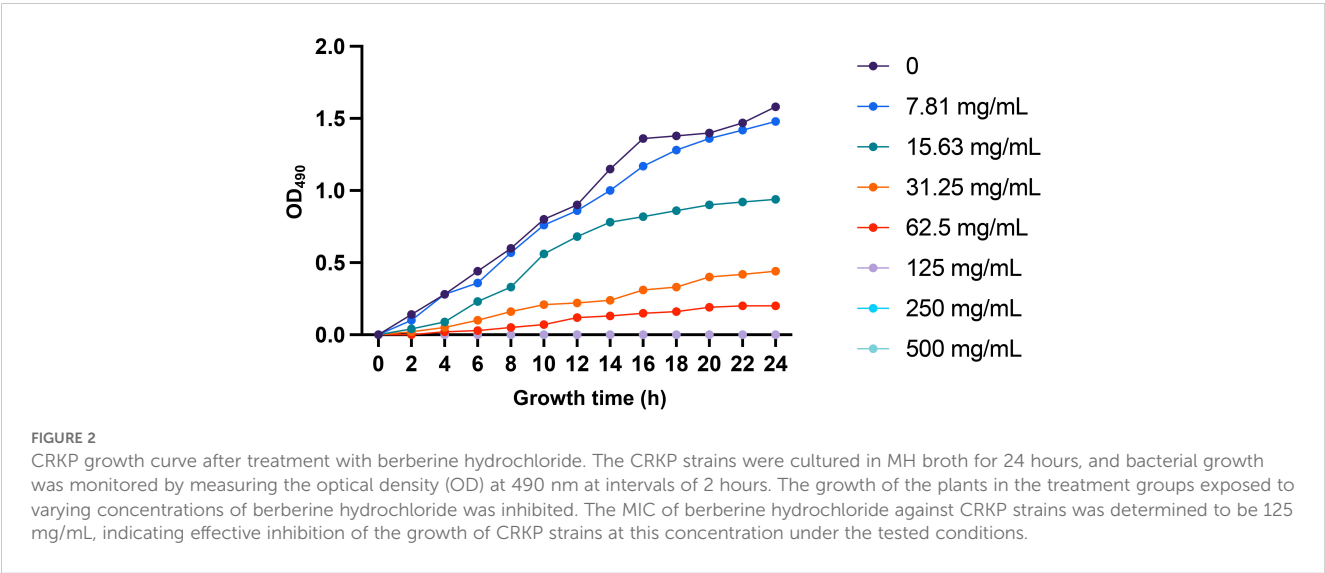


TABLE 5 Multivariate binary logistic regression analysis for poor prognosis in patients with CRKP infection.

Risk factors	Wald χ^2	P	OR	95% CI
Traditional Chinese medicine treatment	3.941	0.047	4.025	1.018-15.918
Respiratory diseases	5.363	0.021	0.099	0.014-0.701
Venous cannula	18.253	<0.001	0.058	0.016-0.214
Third-generation cephalosporin enzyme inhibitor treatment	4.141	0.042	0.218	0.05-0.945
Intercept	2.493	0.114	2.197	0.827-5.839

The dependent variable is poor prognosis; McFadden $R^2 = 0.420$; Cox & Snell $R^2 = 0.400$; Nagelkerke $R^2 = 0.568$.

In our study, ~84% of bloodstream infections (71.28% ICU, 12.77% hematology) originated in high-risk units, reflecting the vulnerability of critically ill patients with prolonged hospitalization, immunosuppression, and invasive interventions. While established risk factors (carbapenem use, ICU admission, invasive procedures) align with prior studies (Li et al., 2020; Yuan et al., 2020; Zhang et al., 2021), we identified novel prognostic determinants: respiratory comorbidities, intravenous catheterization, and prior third-generation cephalosporin/enzyme inhibitor use independently predicted poor outcomes (Hussein et al., 2013; Brennan et al., 2014), whereas traditional Chinese medicine adjunctive therapy significantly improved prognosis. Our findings suggest the need for adjunctive therapy trials to evaluate the immunomodulatory or anti-biofilm effects of traditional Chinese medicine's in CRKP management.

Recent studies have focused on the antibacterial properties of traditional Chinese medicine owing to its low propensity for drug resistance and broad-spectrum antibacterial effects across multiple target sites (Wojtyczka et al., 2014; Li et al., 2021). Meanwhile, treatment with traditional Chinese medicine, either alone or in combination with Western medicine, is emerging as a novel approach to combat drug-resistant bacteria. Previous studies have highlighted the synergistic effects of combining berberine hydrochloride (commonly known as berberine) with antibiotics (Wojtyczka et al., 2014; Zhou et al., 2023). According to our study results, berberine hydrochloride exhibited significant antibacterial activity *in vitro*, with an MIC of 125 mg/mL against CRKP isolates, suggesting its potential for clinical use in anti-CRKP treatment. However, in-depth research in this direction is warranted to explore and develop additional Chinese medicine treatment options for patients with multidrug-resistant CRKP infections. Overall, our study highlights the potential of traditional Chinese medicine as a valuable resource for addressing the challenges posed by drug-resistant bacteria.

Limitations of the study

Despite several significant findings, a few limitations of the present study should be acknowledged. First, the relatively small sample size may have affected the analysis of risk factors. Second, the underlying mechanism by which berberine hydrochloride affects CRKP strains, particularly multidrug-resistant strains, still needs to be investigated. Therefore, future research with larger sample sizes and focused mechanistic exploration is warranted to

address these limitations, thereby providing a more comprehensive understanding of CRKP infections and their treatment options.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Medical Ethics Committee of the hospital (No. 2019-KL-090-01) and was conducted in compliance with ethical, legal, and regulatory norms. The requirement for consent was waived by the Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University because of the retrospective nature of the review. The informed consent form (ICF) was not required because it was a retrospective study, and the personal data of the involved subjects were anonymized and maintained with confidentiality. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CZ: Visualization, Writing – original draft, Conceptualization, Data curation, Formal Analysis, Investigation, Project administration, Software, Writing – review & editing. SL: Investigation, Writing – review & editing, Data curation, Methodology, Formal Analysis, Software. ZL: Data curation, Formal Analysis, Investigation, Software, Writing – review & editing. XY: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

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Characterization of FosA13, a novel fosfomycin glutathione transferase identified in a *Morganella morganii* isolate from poultry

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Background: *M. morganii* is a species of the genus *Morganella* in the family *Enterobacteriaceae*. This species primarily causes infections of postoperative wounds and the urinary tract. Some isolates of *M. morganii* exhibit resistance to multiple antibiotics due to multidrug resistance traits, complicating clinical treatment; thus, there is a growing need to elucidate the resistance mechanisms of this pathogen.

Methods: A total of 658 bacterial strains were isolated from anal fecal swabs from poultry and livestock and from the surrounding environment in Wenzhou, China, via plate streaking. The full genome sequences of the bacteria were obtained via next-generation sequencing platforms. The standard agar dilution method was employed to determine the minimum inhibitory concentrations (MICs) of various antimicrobial agents. The resistance gene (*fosA13*) of the isolate was identified using the Comprehensive Antibiotic Resistance Database (CARD) and confirmed via molecular cloning. The FosA13 protein encoded by the novel resistance gene *fosA13* was expressed with the vector pCold I, and its enzyme kinetics parameters were characterized. The genetic background and evolutionary process of the sequence of this novel resistance gene were analyzed by means of bioinformatics methods.

Results: In this study, we identified a new chromosomally encoded fosfomycin resistance gene, designated *fosA13*, from the *M. morganii* isolate DW0548, which was isolated from poultry on a farm in Wenzhou, China. Compared with the control strain (pUCP19/DH5 α), the recombinant strain carrying *fosA13* (pUCP19-*fosA13*/DH5 α) presented a fourfold increase in the MIC value for fosfomycin. The enzyme kinetics data of FosA13 revealed effective inactivation of fosfomycin, with a k_{cat}/K_m of $(1.50 \pm 0.02) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Among functionally characterized

resistance proteins, FosA13 presented the highest amino acid (aa) homology (55.6%) with FosA. FosA13 also contained essential functional residues of FosA proteins. The isolate *M. morganii* DW0548 presented high MIC values ($\geq 8 \mu\text{g/mL}$) for 5 classes of antimicrobials, namely, aminoglycosides, β -lactams, quinolones, tetracycline, and chloramphenicol, but only two functionally characteristic antimicrobial resistance genes (ARGs) have been identified in the complete genome: a β -lactam resistance gene (*bla*_{DHA-16}) and a phenol resistance gene (*catII*). These findings indicate that in addition to the novel resistance gene identified in this work, other uncharacterized resistance mechanisms might exist in *M. morganii* DW0548.

Conclusion: A novel chromosomal fosfomycin resistance gene, *fosA13*, was identified in an animal *M. morganii* isolate, and its enzymatic parameters were characterized. This protein shares the highest aa identity of 55.6% with the functionally characterized protein FosA and has all the essential functional residues of FosA proteins. Exploring more antimicrobial resistance mechanisms of this pathogen would help clinicians choose effective drugs to treat infectious diseases in animal husbandry and clinical practice and facilitate the development of methods to prevent the spread of resistance between bacteria of different species.

KEYWORDS

Morganella morganii, resistance gene, *fosA13*, fosfomycin glutathione transferase, kinetic parameter

Introduction

In 1969, a natural antibiotic named fosfomycin was first discovered in the fermentation broth of *Streptomyces* (Hendlin et al., 1969). Although some species can produce fosfomycin, its concentrations are generally low. Fosfomycin exhibits bactericidal properties against various bacteria, including both Gram-negative and Gram-positive bacteria such as staphylococci (Raz, 2012). Fosfomycin was a decommissioned antibiotic, however, given the increasing prevalence of multidrug-resistant uropathogens, the limited treatment options available, and the lack of new antibiotics, older antibiotics need to be reevaluated. Owing to its unique bactericidal mechanism and physicochemical properties, fosfomycin has the advantages of no cross-resistance, strong antibacterial activity, a wide tissue distribution, and synergistic bactericidal effects when used in combination with other drugs, and was defined by the World Health Organization (WHO) as a “vital” antibiotic (Collignon et al., 2016), which has attracted the interest of many clinicians. With an increase in the frequency of clinical fosfomycin use, resistance to fosfomycin has also increased in some bacteria, such as *Acinetobacter*, *Vibrio fischeri*, *Chlamydia trachomatis*, and so on (Silver, 2017).

The earliest case of fosfomycin resistance dates back to 1977. Since then, there have been epidemics of drug-resistant strains in all countries worldwide (Aghamali et al., 2019). Although the mechanism of action and the structure of fosfomycin are unique, which made the cross-

resistance uncommon (Falagas et al., 2020), with the increased use of fosfomycin, bacterial resistance to it has also increased rapidly. Data show that the use of fosfomycin in the treatment of urinary tract infections caused by *Escherichia coli*, as well as in the treatment of some uropathogen infections, leads to an increase in fosfomycin resistance (Jiang et al., 2015).

Several categories of fosfomycin drug resistance mechanisms have been characterized (Karageorgopoulos et al., 2012). These mechanisms involve reducing drug absorption, altering drug binding targets, and inactivating fosfomycin. Resistance to fosfomycin is typically associated with the inactivation of fosfomycin by modifying enzymes (such as *fosA*, *fosB*, *fosC*, and *fosX*) (Yang et al., 2019; Findlay et al., 2023), and kinases (*fomA* and *fomB*) (Kobayashi et al., 2000). FosA is a dimeric glutathione S-transferase (GST) that catalyzes the binding of glutathione to fosfomycin with the help of Mn^{2+} and K^{+} ions, inactivating fosfomycin, and it can be encoded on either a plasmid or a chromosome (Suárez and Mendoza, 1991). FosA was first found to be present in the plasmid Tn2921 transposon of *Serratia marcescens*, and it is predominantly present in Enterobacteriaceae, *Pseudomonas* spp (Biggel et al., 2021). The FosA proteins are a number of metalloenzymes able to disrupt the epoxide ring of fosfomycin. It depends on manganese (II) and potassium as cofactors, and glutathione (GSH) as a nucleophilic molecule (Mattioni Marchetti et al., 2023). The *fosA* genes are commonly distributed in *Providencia stuartii*, *Providencia rettgeri*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*,

Serratia marcescens, *Enterobacter aerogenes* and *Enterobacter cloacae*, however, they are rarely reported in *Citrobacter freundii*, *Proteus mirabilis* and *Acinetobacter baumannii* (Zurfluh et al., 2020). FosB is an L-cysteine thioltransferase that inactivates fosfomycin via the nucleophilic addition of thiols to fosfomycin with the help of Mg^{2+} (Michalopoulos et al., 2011). FosC is a protease inactivating fosfomycin by adding a phosphate group to it by using ATP as a substrate (Garcia et al., 1995). FosX is a Mn^{2+} -dependent epoxide hydrolase that inactivates fosfomycin by adding a hydroxyl group to the fosfomycin C1 position and opening its epoxide ring, using water as a substrate (Rigsby et al., 2005). FomA and FomB are kinases involved in the degradation of fosfomycin, and the role of these kinases may be to protect fosfomycin producers from fosfomycin (Kobayashi et al., 2000). Chromosomal mutations can affect the transport function of the cell membrane, leading to reduced intracellular levels of fosfomycin. Two transport systems for the uptake of fosfomycin into cells, which involve glycerol-3-phosphate transporter protein (GlpT) and hexose phosphate transporter protein (UhpT), are present in *E. coli* (Takahata et al., 2010). Altering the drug's target of action is another mechanism of fosfomycin resistance. Among Gram-positive bacteria, the affinity between the MurA protein and fosfomycin in *S. aureus* is reduced by mutations in the *murA* gene (Xin et al., 2022); however, elevated expression levels of this gene can lead to bacterial resistance to fosfomycin (Raina et al., 2021).

M. morganii, a pathogen that was first isolated from pediatric fecal cultures by Morgan et al. in 1906 (Morgan and de, 1906), is a parthenogenetic anaerobic rod-shaped Gram-negative enteric bacterium that produces virulence factors such as hemolysin and causes urinary tract wound infections, and the risk of infection by *M. morganii* has been highlighted in many epidemiological data. Clinical disease caused by multidrug-resistant (MDR) or even extensively drug-resistant (XDR) *M. morganii* often results in treatment failure. Studies have shown that the development of intrinsic and acquired multidrug resistance is of concern as the prevalence of *M. morganii* infections increases, necessitating the global identification of *M. morganii* as a major pathogen (Liu et al., 2016a; Bandy, 2020a; Li et al., 2023). Rising rates of *M. morganii* infections are a reminder not only of the need for increased precautions in public areas but also of the need to include this microorganism in the differential diagnosis list in the clinical setting (Bandy, 2020).

In this work, we report a novel chromosomally encoded fosfomycin gene, *fosA13*, in the isolate DW0548, which was isolated from a farm animal. DW0548 was subjected to whole-genome sequencing for genome-wide characterization. To determine the function of the *fosA13* gene, molecular cloning was performed. The protein encoded by *fosA13* was expressed, and its enzyme kinetics were also analyzed.

Materials and methods

Origin of bacterial strains and identification of species

To analyze the drug resistance status of bacteria isolated from animals and the environment of the animal farms, 658 bacterial

strains were obtained from the poultry and livestock anal fecal swabs, and sewage and soil of the animal farms in Wenzhou, China. An *fosA*-like gene, designated *fosA13*, was found in an isolate from poultry named DW0548. Bacterial species identification was performed via 16S rRNA gene homology and genome-wide average nucleotide identity (ANI) analyses. Table 1 lists the plasmids and strains used in this study.

Antibiotic susceptibility testing of the bacteria

The MICs of the antimicrobials were determined via the agar dilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2024). Medium with glucose-6-phosphate (G6P) at a constant concentration of 25 μ g/mL was used when the MIC of fosfomycin was tested. *E. coli* ATCC 25922 was used as a quality control. Table 2 shows the MIC data for the 25 antibiotics from the six antibacterial categories.

Whole-genome sequencing and functional analysis

The bacteria were cultured in liquid LB medium and incubated at 37°C for 16 h. Total DNA was extracted from the bacteria with the Genaray Genomic DNA Small Volume Preparation Kit (Shanghai Genaray Biotech Co., Ltd., Shanghai, China). Whole-genome sequencing was completed on the Illumina NovaSeq and PacBio RS II platforms at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The Illumina short reads and the PacBio long reads were assembled using MEGAHIT v1.2.9 (Li et al., 2016) and Tricycler v0.5.1 (Wick et al., 2021), respectively. Using Pilon v1.24, the quality of the genome sequence from PacBio sequencing was corrected by mapping the Illumina short reads onto the PacBio read assembly (Walker et al., 2014). The open reading frames (ORFs) were predicted with Prokka v1.14.6 (Seemann, 2014). The functions of the predicted proteins were annotated by searching the ORFs against the NCBI nonredundant protein database with DIAMOND v2.0.11 (Buchfink et al., 2021). The promoter region of a gene was predicted using the BPROM tool (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>). Annotation of drug resistance genes was conducted by using Resistance Gene Identifier v5.2.0 (RGI) and the Comprehensive Antibiotic Resistance Database (CARD) database (<https://github.com/arpcard/rgi>) (McArthur et al., 2013). Homology analysis of the 16S rRNA gene from the target genome was performed by comparison with the 16S ribosomal RNA sequence database in NCBI (McArthur et al., 2013). The ANI was computed using FastANI v1.33 (Jain et al., 2018). Calculation of digital DNA–DNA hybridization (dDDH) values was performed on the basis of the Type strain Genome Server (TYGS) online database (Type Strain Genome Server) (Lian et al., 2021). Multiple sequence alignment of *fosA13*, *fosA*, *fosA2*, and other related genes was performed with MAFFT v7.487 (Katoh and Standley, 2013). A neighbor-joining (N-J)

TABLE 1 Bacteria and plasmids used in this work.

Bacteria and plasmids	Description	Source
DW0548	The wild type of <i>Morganella morganii</i> DW0548	Chicken
DH5α	<i>E. coli</i> DH5α as a host cell for cloning of the <i>fosA13</i> gene	Our laboratory collection
pUC19	Cloning vector for the PCR product of <i>fosA13</i> with its upstream promoter region, AMP ^r	Our laboratory collection
ATCC25922	<i>E. coli</i> ATCC 25922 as quality control for MIC testing	Our laboratory collection
BL21	<i>E. coli</i> BL21 as a host cell for the expression of FosA13	Our laboratory collection
pColdI	Expression vector for the PCR product of the ORF of the <i>fosA13</i> gene, AMP ^r	Our laboratory collection
pUCP19- <i>fosA13</i> /DH5α	The DH5α cell carrying recombinant plasmid pUCP19- <i>fosA13</i>	This research
pCold I- <i>fosA13</i> /BL21	The BL21 cell carrying the recombinant plasmid pCold I- <i>fosA13</i>	This research

^r, resistance; AMP, ampicillin; ORF, open reading frame.

phylogenetic tree including FosA13 and other functionally characterized fosfomycin resistance enzymes was constructed with MEGA11 (Kumar et al., 2018). The phylogenetic tree of FosA13 with other Fos proteins was visualized with the online website iTOL (iTOL: Interactive Tree Of Life) (Letunic and Bork, 2021).

Molecular cloning of the identified resistance gene

Referring to a previous publication (Zhao et al., 2023), primers to clone the predicted resistance gene with its promoter region and the primers to clone the open reading frame (ORF) of the gene were designed. The PCR products were amplified and then inserted into the vectors pUCP19 and pCold I, respectively, via the DNA ligation kit Ver.2.1 (Takara Bio, Inc., Dalian, China). The recombinant plasmids pUCP19-*fosA13* and pCold I-*fosA13* were transformed into *E. coli* DH5α and *E. coli* BL21, respectively. The inserted sequences in the recombinants were verified by Sanger sequencing. Table 3 shows the primer sequences and details.

Expression and purification of the recombinant FosA13 protein

The methods used to express and purify the recombinant FosA13 protein were mainly based on a previous publication (Gao et al., 2022). In brief, the recombinant strain (pCold I-*fosA13*/BL21) was cultured in LB broth. When the OD value of the culture reached between 0.45 and 0.55, the expression of the protein was induced by 1 mM isopropyl-β-dithiogalactopyranoside (IPTG). Bacteria were harvested by centrifugation (4,000 × g, 10 min) at 4°C, resuspended in 5 mL of nondenaturing lysis buffer, and fragmented by ultrasonication for 10 min at 4°C. After centrifugation, the supernatant containing the recombinant protein was collected. The recombinant protein was purified with BeyoGold His-tag purification resin using a nondenaturing elution solution from a His-tag protein purification kit (Beyotime, Shanghai, China). The His-tag of the recombinant protein was removed by enterokinase (EK enzyme).

The purity of the protein was determined via SDS–PAGE, and the protein concentration was subsequently determined using a BCA protein concentration assay kit (Beyotime, Shanghai, China).

Enzyme kinetics studies of FosA

The kinetic parameters of purified FosA13 with fosfomycin were analyzed via high-performance liquid chromatography (HPLC) using a Thermo Scientific Accela HPLC system (Thermo Fisher Scientific, Inc., China) with a 100 μL final reaction volume at 37°C. The 100 μL reaction system consisted of 10 mM GSH, 250 μM MnCl₂, 100 mM KCl, and 100 mM Tris-HCl, and gradient concentrations of fosfomycin (0, 25, 50, 100, 200, 400, and 800 μM) were added in a volume of 10 μL. After the reaction system was preheated for 20 minutes, the purified FosA13 protein was added. The reaction was conducted for 5 minutes, and the assay was carried out on the analysis system. The mobile phases were a mixture containing K₂HPO₄ (200 mM), KH₂PO₄ (200 mM), acetonitrile and methanol at a percentage volume ratio of 1.68: 78.32: 10: 10 (Arca et al., 1990; Bernat et al., 1997; Bernat et al., 1999). The steady-state kinetic parameters *k*_{cat} and *K*_m were nonlinearly regressed against the initial reaction rate via the Michaelis–Menten equation in Prism (version 8.0.2) software (GraphPad software, CA, United States). The value is the average of three independent measures.

Nucleotide sequence accession numbers

The GenBank accession numbers for the novel fosfomycin *fosA13* gene, the plasmid pMMDW0548 and the chromosome sequences of DW0548 are PQ600006, CP173708 and CP173707, respectively.

Results and discussion

Discovery of a new drug resistance gene

To clarify the mechanisms of antimicrobial resistance in bacteria isolated from animals and the environment, we sequenced 658

TABLE 2 The MIC results of 25 antimicrobials for 5 strains (μg/mL).

Drug class	Antimicrobials	ATCC25922	DH5α	pUCP19/DH5α	pUCP19- <i>fosA13</i> /DH5α	<i>M. morganii</i> DW0548
Aminoglycosides	Gentamicin	0.5	8	0.5	/	1
	Tobramycin	0.5	0.5	0.5	/	0.5
	Streptomycin	4	2	2	/	8
	Kanamycin	8	8	8	/	8
	Speptomycin	8	64	8	/	32
	Paromomycin	4	4	8	/	8
	Neomycin	1	16	8	/	2
	Sisomicin	≤1	≤1	≤1	/	≤1
	Amikacin	≤2	≤2	≤2	/	≤2
	Netilmicin	≤0.125	≤0.125	≤0.125	/	≤0.125
	Ribostamycin	4	≤2	≤2	/	4
β-Lactams	Cefazolin	2	1	16	/	512
	Cefothiofene	32	≤4	64	/	>1024
	Cefoxitin	4	4	4	/	16
	Cefuroxime	16	16	16	/	16
	Ceftazidime	0.5	1	1	/	0.5
	Cefotaxime	0.5	≤0.06	0.125	/	0.125
	Ceftriaxone	0.125	8	0.125	/	0.0625
	Cefepime	0.03	0.015	0.0625	/	0.03
Quinolones	Levofloxacin	≤0.05	≤0.05	≤0.05	/	≤0.05
	Nalidixic acid	64	32	64	/	64
Tetracycline	Tetracycline	1	2	1	/	32
Phosphonic acid derivative	Fosfomycin	0.5	0.5	0.5	2	2
Amphenicols	Chloramphenicol	16	16	16	/	64
	Florfenicol	4	8	4	/	4

bacterial isolates isolated from poultry and livestock anal fecal swabs and environmental samples from animal farms in Wenzhou, China. Annotation of genomic data revealed resistance genes against different antibiotics. Notably, among the predicted genes were numerous putative fosfomycin resistance genes, including but not limited to homologs of *fosA*, *fosB*, *fosC*, and *fosX*. These genes shared less than 80.0% aa sequence identity with functionally characterized fosfomycin resistance genes. Some of these genes, including the *fosA2*, *fosA5*, *fosB*, *fosLC2* and *fosL1* homologs, were randomly selected and cloned, and their resistance functions were determined. Finally, a gene homologous to *fosA2* (designated *fosA13* in this work) that conferred resistance to fosfomycin was identified, and it was encoded in the chromosome of an isolate named DW0548.

Cloning and phenotyping of the novel resistance gene

To confirm the resistance function of this gene, the ORF with its promoter region was cloned into the vector pUCP19, and the recombinant plasmid containing *fosA13* was transformed into *E. coli* DH5α cells. The transformant strain carrying *fosA13* (pUCP19-*fosA13*/*E. coli* DH5α) showed a 4-fold increase in the MIC value to fosfomycin (2 μg/mL) compared with that of the control strain (pUCP19/DH5α, 0.5 μg/mL) (Table 2). The MICs of the previously cloned fosfomycin resistance genes varied. Compared with the recipients, the cloned *fosY* (Chen et al., 2022) and *fosA6* (Guo et al., 2016a) genes increased the MIC levels by 16- and 32-fold, respectively, whereas *fosI* (Pelegrino et al.,

TABLE 3 Primers for cloning the *fosA13* gene.

Primer ^a	Sequence(5' -3') ^b	Restriction endonuclease	Vector	Annealing temperature (°C)	Amplicon size (bp)
pro- <i>fosA13</i> -F	TCAGTTCCATAACAGTGAGAAAGCC	/	pUCP19	58	879
pro- <i>fosA13</i> -R	TAGCAGTGTCTCCGTAAGATAAGGG	/	pUCP19	59	879
orf- <i>fosA13</i> -F	CGGGGTACCGACGACGACGACAAGATGTTAACAGGAATGAATCATCTG	KpnI	pColdI	54	451
orf- <i>fosA13</i> -R	CCCAAGCTTGATAAGGGTTATCCCATCTTACAC	HindIII	pColdI	55	451

^aPrimers with “pro” were used to clone the *fosA13* gene with its promoter region, and primers with “orf” were used to clone the ORF of the *fosA13* gene.

^bThe underline sequences represent the restriction sites and their protective bases.

2016) and *fosA7* (Rehman et al., 2017) increased the MIC levels by 128- and >256-fold, respectively (Supplementary Table S1).

Classification and genome characterization of the isolate DW0548

16S rRNA gene homology analysis revealed that the 16S rRNA gene of the isolate DW0548 shared the highest similarity (96.0% coverage and 99.0% identity) with that of *M. morganii* M11 (NR_028938.1). In addition, ANI analysis of all 759 *Morganella* genomes in the NCBI database revealed that 78 of these genomes had $\geq 95.0\%$ ANI (the threshold value to define a bacterial species) with the isolate DW0548 genome. Seventy-three of these genomes were from the *M. morganii* genomes. The result of the digital DNA–DNA hybridization (dDDH) analysis revealed that the isolate DW0548 presented the highest dDDH value (75.7%) with *M. morganii* NBRC 3848, which was greater than the threshold (70.0%) for classifying a bacterial species. Therefore, on the basis of the results above, the isolate DW0548 was ultimately included in the species *M. morganii* and was thus designated *M. morganii* DW0548.

The whole genome of *M. morganii* DW0548 consists of a chromosome and a plasmid named pMM548. The chromosome size was approximately 4.31 Mb, and the average GC content was 50.35%, with 4,380 coding sequences (CDSs). The plasmid was 96,349 bp in length with the average GC content of 50.45%, and it encoded 109 CDSs (Table 4).

The resistance profile of *M. morganii* DW0548

The result of the susceptibility test demonstrated that *M. morganii* DW0548 had high MICs (≥ 8 µg/mL) for 11 of the 25 antimicrobials tested, which included members of 5 classes of antimicrobials, with 4 aminoglycosides (streptomycin, kanamycin, spectinomycin and paromomycin), 4 β -lactams (cefazolin, cefothiophene, cefoxitin and cefuroxime), 1 quinolone (naphridixic acid), tetracycline, and chloramphenicol (Table 2).

In examining the relationship between the drug resistance phenotype and genotype, we found that even though the bacterium presented high MICs for antimicrobials from the 5 classes tested, only two genes (a β -lactam resistance gene, *bla*_{DHA-16}, and a phenicol resistance gene, *catII*), which shared $\geq 80\%$ aa similarity with

functionally characterized antimicrobial resistance genes (ARGs), were identified. No aminoglycoside, tetracycline or quinolone resistance genes were found in the whole-genome sequence (Supplementary Table S2). Similar to the isolate DW0548, other *M. morganii* strains have been reported to be resistant to β -lactams, aminoglycosides, tetracyclines, fluoroquinolones, fosfomycin, and other types of antibiotics (Liu et al., 2016).

Homology analysis of FosA13 with the other FosA proteins

A comparison of FosA13 with these functionally characterized proteins in the CARD revealed that it had the greatest aa sequence similarities with FosA (55.6%), followed by FosA2 (55.2%), FosA3 (55.2%), FosA4 (55.2%), FosA5 (55.2%), FosA6 (55.2%), FosA7 (52.9%), FosA7.5 (52.9%), and FosA8 (52.9%). Sixteen function and structure essential residues of FosA (Beharry and Palzkill, 2005a) are conserved in FosA13. Half of them act as ligands for Mn²⁺ (His7, His64 and Glu110) and K⁺ (Ser94, Ser98 and Glu95) and ligands (Arg119 and Tyr100, within the hydrogen-bonding site of fosfomycin) involved in FosA binding to fosfomycin, and the other half of the residues (W34, Y39, W46, C48, Y65, D103, H107, Y128) were located in the putative fosfomycin/GSH binding channel. Although FosA13 shares only about 50–60% identity with these functionally characterized FosA proteins, the active site residues essential for FosA function remain unchanged (Figure 1). Further analysis of the evolutionary relationship between FosA13 and different glutathione transferases revealed that FosA13 was most similar to FosA2 and formed a new branch (Figure 2).

Kinetic parameters of FosA13

The *fosA13* gene is 432 bp in length and encodes a 143 aa protein (FosA13). The predicted molecular weight of the mature glutathione-S-transferase is 16.48 kDa, with a pI of 6.08. The purified FosA13 has the ability to hydrolyze fosfomycin, with a K_m of 0.427 ± 0.007 µM, k_{cat} of 6.43 ± 0.04 s⁻¹ and k_{cat}/K_m of $(1.50 \pm 0.02) \times 10^4$ M⁻¹ s⁻¹. In terms of enzyme kinetics, compared with the other two FosA proteins, FosA13 showed 6-fold lower hydrolytic activity against fosfomycin than that of FosA (Beharry and Palzkill, 2005b) (k_{cat}/K_m of 1.5×10^4 M⁻¹ s⁻¹ vs. 9.0×10^4 M⁻¹ s⁻¹) and approximately 5-fold lower than that of FosA6 (Guo et al., 2016) (k_{cat}/K_m of 1.5×10^4 M⁻¹ s⁻¹ vs. 0.3×10^4 M⁻¹ s⁻¹).

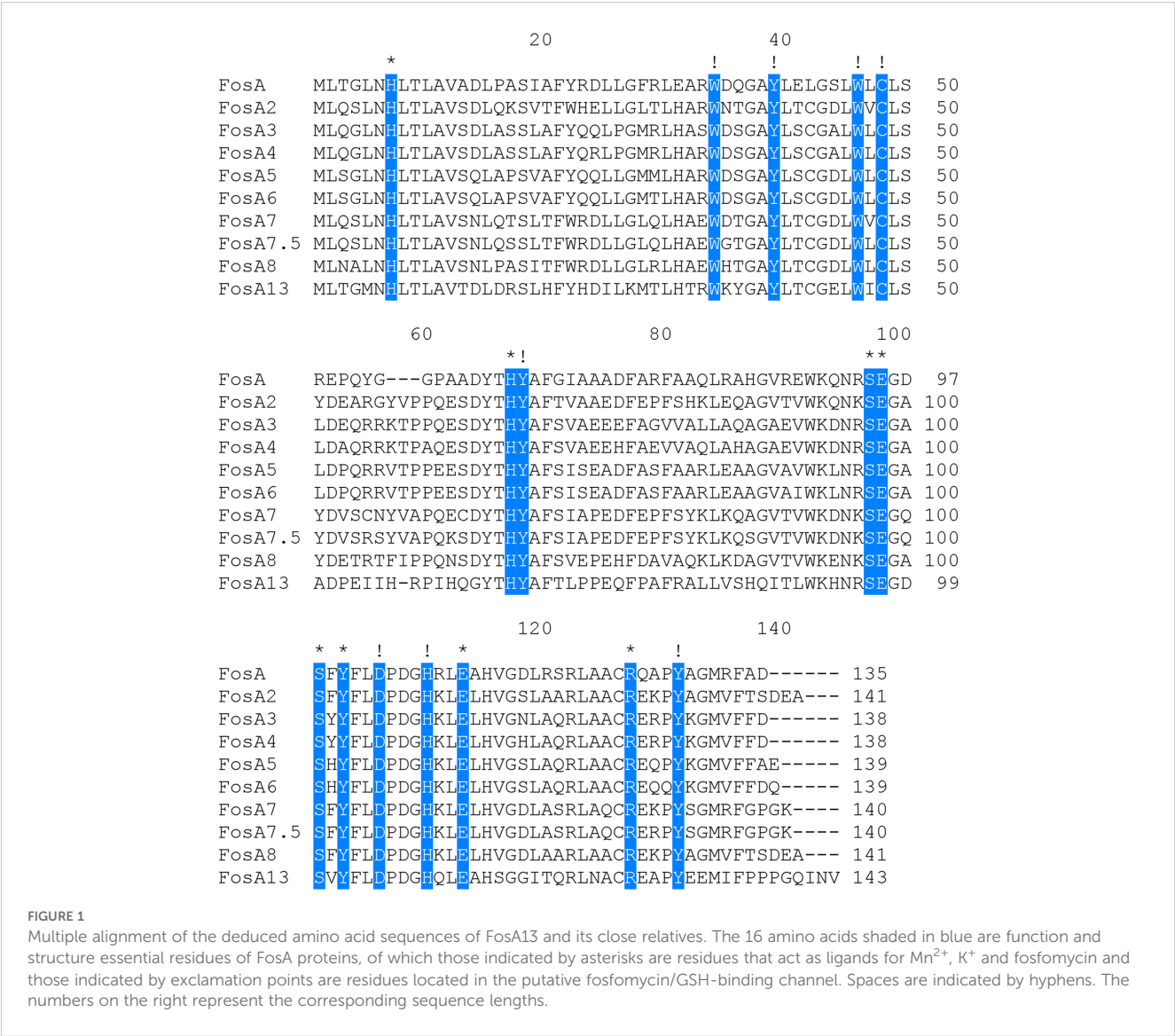
TABLE 4 General features of the *fosA13* genome.

	Chromosome	pMMDW0548
Size (bp)	4,313,176	96,349
GC content (%)	50.35	50.45
Predicted coding sequences (CDSs)	4,380	109
Known proteins	3,151	38
Hypothetical proteins	1,229	71
Protein coding (%)	96.31	99.08
Average ORF length (bp)	868.4	820.7
Average protein length (aa)	288.6	262.3
tRNAs	81	0
rRNA operons	(16S-23S-5S) × 6 (16S-23S-5S-5S) × 1	0

Distribution and context of the *fosA13*-like genes

To investigate the distribution of *fosA13*-homologous genes, the nucleotide sequence of *fosA13* was used as a query to search for similar genes in the NCBI nucleotide database. As a result, a total of 82 similar genes with similarities between 87.3% and 100.0% were obtained, all of which were derived from the *M. morganii* genomes (Supplementary Table S3). Among these 82 genes, only one had a similarity of 87.3%, and all the others had similarities of ≥ 92.13%. The gene context of the *fosA13*-homologous genes was further analyzed. The 20-kb sequences with the *fosA13*-homologous genes at the center were intercepted and clustered. Finally, these 83 20-kb fragments (including the gene identified in this study) were grouped into 13 clusters with a similarity threshold of 90.0%. The gene context of 13 sequences consisting of one from each of the 13 clusters was compared (Figure 3, Supplementary Table S3).

As mentioned above and illustrated in Figure 3, the *fosA13* and *fosA13*-homologous genes and their surrounding sequences were



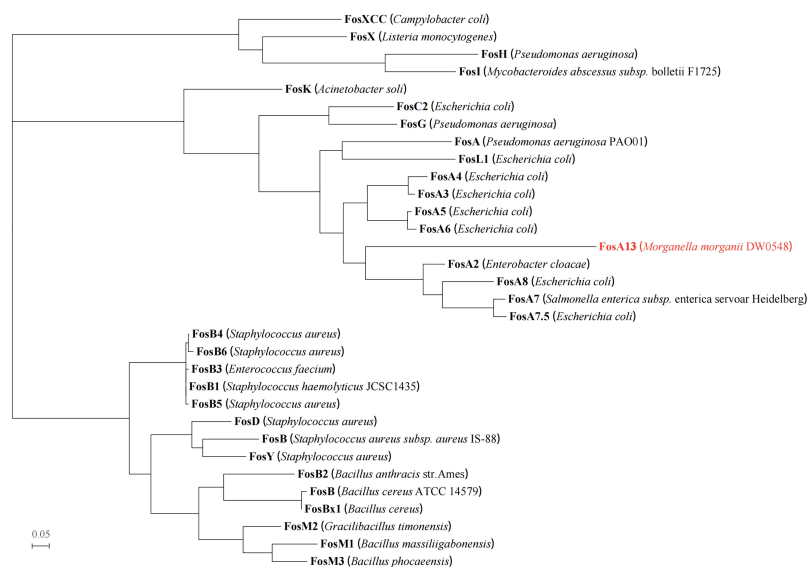


FIGURE 2 Phylogenetic tree showing the relationship of FosA13 with other functionally characterized proteins (with identities between 20.0% and 60.0%). FosA13 is highlighted in red. The other proteins include FosA (AAG04518.1), FosA2 (ACC85616.1), FosA3 (AEG78825.1), FosA4 (BAP18892.1), FosA5 (AJE60855.1), FosA6 (AMQ12811.1), FosA7 (KKE03230.1), FosA7.5 (ANQ03635.1), FosA8 (QE122965.1), FosB (EHS19134.1, *S. aureus*), FosB (AAP08996.1, *B. cereus*), FosB1 (BAE05988.1), FosB2 (AAP27834.1), FosB3 (ADX95999.1), FosB4 (ALM24139.1), FosB5 (ALN12426.1), FosB6 (ALM24145.1), FosBx1 (QLF01382.1), FosC2 (BAJ10053.1), FosD (BAG12271.1), FosG (RTB44598.1), FosH (ADF48907.1), FosI (AFJ38137.1), FosK (BAO79518.1), FosL1 (QHR93773.1), FosM1 (DAC85639.1), FosM2 (DAC85640.1), FosM3 (DAC85641.1), FosX (CWV56762.1), FosXCC (AIF29598.1), and FosY (QTE33800.1).

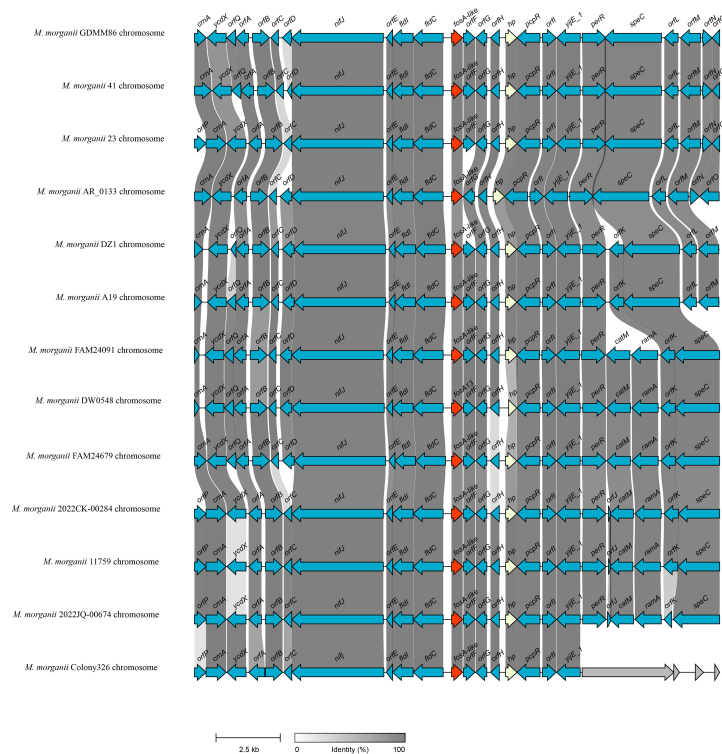


FIGURE 3 Genetic environment of *fosA13* and *fosA13*-homologous genes. The *fosA* genes are in red. *orfA*, DUF523 domain-containing protein; *orfB*, HAD family hydrolase; *orfC*, DUF5339 domain-containing protein; *orfD*, GNAT family N-acetyltransferase; *orfE*, DUF3343 domain-containing protein; *orfF*, glyoxalase; *orfG*, carboxymuconolactone decarboxylase family protein; *orfH*, DMT family protein; *orfI*, DMT family transporter; *orfJ*, aminoglycoside 6'-acetyltransferase; *orfK*, SM11/KNR4 family protein; *orfL*, GNAT family N-acetyltransferase; *orfM*, SDR family NAD(P)-dependent oxidoreductase; *orfN*, MerR family transcriptional regulator; *orfO*, methyl-accepting chemotaxis protein; *orfP*, cytosine permease; *orfQ*, glyoxalase/bleomycin resistance/dioxygenase family protein.

relatively conserved in the *M. morganii* genomes. Further structural analysis revealed that no mobile genetic element (GME) was present in their flanking regions. Upstream of *fosA13* are genes encoding proteins related to *nifH* [ferredoxin (flavodoxin) oxidoreductase], *fldI* [phenyllactate dehydratase activator] and *fld* [(R)-phenyllactyl-CoA dehydratase beta subunit], whereas downstream of *fosA13* are genes related to *pcpR* (PCP degradation transcriptional activation protein), *yijE_1* (putative cystine transporter YijE_1) and *perR* (HTH-type transcriptional regulator PerR). However, many other *fos* genes, such as *fosC2* (Wachino et al., 2010), *fosA3* (Wachino et al., 2010), *fosA5* (Ma et al., 2015), *fosA6* (Guo et al., 2016) and *fosA8* (Poirel et al., 2019), are related to MGEs and are encoded on plasmids.

Conclusion

This paper presents the discovery of a novel chromosomal fosfomycin resistance gene, designated *fosA13*, from an animal *M. morganii* isolate. Although *fosA13* encodes a protein that shares less than 80% aa similarity with functionally characterized FosA proteins, the function and structure essential residues of these proteins are conserved within it. Many *fosA*-type genes are located on plasmids of different bacterial species. These genes are easily captured by mobile genetic elements and transmitted between bacteria of different species by means of horizontal gene transfer, which results in widespread resistance. Identifying more resistance mechanisms would greatly benefit the treatment of bacterial infections in the clinic and the monitoring of resistance transmission.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

This study used strains obtained from poultry, livestock anal fecal swabs, and the environment in animal farms in Wenzhou, China. The owners of the farms were written informed of the study and expressed approval for sampling of animals. The studies involving human participants and animals were reviewed and approved by the Animal Welfare and Ethics Committee of Wenzhou Medical University, Zhejiang Province, China (Protocol number: wyd2021-0323).

Author contributions

RZ: Writing – original draft, Writing – review & editing, Data curation, Investigation, Supervision, Validation, Visualization. YY: Writing – original draft, Writing – review & editing, Data curation, Investigation, Supervision, Validation, Visualization. LH: Writing – review & editing, Investigation, Supervision, Validation. SC: Writing –

review & editing, Investigation, Supervision, Validation. RH: Writing – review & editing, Investigation, Supervision, Validation. XW: Writing – review & editing, Investigation, Supervision, Validation. DH: Writing – review & editing, Investigation, Supervision, Validation. CS: Writing – review & editing, Data curation, Visualization. JL: Writing – review & editing, Data curation, Visualization. QB: Writing – original draft, Writing – review & editing, Conceptualization, Methodology. YH: Writing – review & editing, Conceptualization, Methodology. PJ: Writing – review & editing, Conceptualization, Methodology. WP: Writing – original draft, Writing – review & editing, Conceptualization, Methodology.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1534084/full#supplementary-material>

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The investigation of molecular epidemiological characteristics and resistance mechanism of tigecycline resistant *Klebsiella pneumoniae* from a large teaching hospital in southwest China, Chongqing

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Background: *Klebsiella pneumoniae* is one of the main pathogens of nosocomial infection, among which carbapenems can be used for multidrug-resistant *Klebsiella pneumoniae*. However, in the past decade, the resistance rate of carbapenem-resistant *Klebsiella pneumoniae* has increased yearly. Tigecycline has good antibacterial activity in treating severe bacterial infections, but the reports of tigecycline resistance are increasing. This study aimed to investigate the mechanism of drug resistance and epidemiological characteristics of tigecycline-resistant *Klebsiella pneumoniae* (TRKP) in a large teaching hospital in southwest China, Chongqing.

Methods: We isolated 30 TRKP strains from this hospital between August 2021 and December 2023. By PCR and sequencing, we examined the presence and mutation rates of genes associated with tigecycline resistance, including *acrR*, *oqxR*, *ramR*, *tmexC*, *tet(x)*, *tet(A)*, *tet(L)*, and *rpsJ*, and performed efflux pump inhibition experiments to verify efflux pump activity. At the same time, real-time RT-PCR was used to detect the expression levels of efflux pump genes (*acrB* and *oqxB*) and *ramA*. To investigate the prevalence trend of TRKP in our hospital, we performed multi-site sequence typing (MLST) analysis.

Results: The mutation rates of *ramR* (73.3%) and *tet(A)* (63.3%) were significant. In efflux pump inhibition experiments, PaßN could reverse the resistance of 29 TRKP strains (96.7%) to tigecycline. Real-time RT-PCR results showed that *acrB* and *ramA* genes were up-regulated in 22 strains, while *oqxB* genes were overexpressed in only 4 strains. MLST analysis showed that these strains could be divided into 25 different ST subtypes, indicating that no outbreak of TRKP occurred in our hospital. In addition, two *tmexCD-torpj* positive strains, ST661 and ST1561, were identified for the first time.

Conclusion: The efflux pump *acrB* and *tet(A)* mutations are the primary mechanisms of resistance to tigecycline-resistant *Klebsiella pneumoniae* at our hospital. The *ramR* mutation can mediate efflux pump activity of *acrB* by up-regulating *ramA* overexpression.

KEYWORDS

resistance mechanism, *Klebsiella pneumoniae*, tigecycline-resistance, *tet(A)*, RND efflux pump

1 Introduction

With the rise of *Klebsiella pneumoniae*, a widely distributed pathogen, critical diseases such as endophthalmitis and bloodstream infections have become common (Lee et al., 2006, 2015; Yang et al., 2021). Carbapenems are often relied on for treating multidrug-resistant (MDR) infections; however, their overuse has resulted in the development of carbapenem-resistant *Klebsiella pneumoniae* (CRKP). Treatments available are increasingly limited, with only last-resort antibiotics like colistin and tigecycline remaining (Sheu et al., 2019). As the first glycylcycline antibiotic, it is a rare effective treatment for difficult-to-treat conditions, particularly those caused by CRKP (Seifert et al., 2018). Like tetracyclines, tigecycline binds reversibly to the 30S ribosomal subunit, disrupting aminoacyl-tRNA function and inhibiting bacterial translation (Pournaras et al., 2016). Tigecycline-resistant strains of *K. pneumoniae* have emerged rapidly since the clinical use of tigecycline, a problem likely exacerbated by the overuse of antibiotics, which has the potential to complicate treatment and pose a significant public health risk.

The current resistance mechanism of tigecycline is mainly related to the overexpression of RND efflux pumps, including AcrAB, OqxAB, MexAB-OprM, and Tmexd-toprJ (Pournaras et al., 2016; Chen et al., 2017; Lv et al., 2020; Avakh et al., 2023). The AcrAB-TolC pump is driven by the global transcriptional activator *RamA* and local inhibitory factor AcrR (Villa et al., 2014). The presence of a mutant in *ramR*, a local inhibitor of *ramA*, leads to elevated *ramA* expression and dysregulation of AcrAB expression, which ultimately leads to tigecycline resistance (Xu et al., 2021). Similarly, inactivation of *OqxR* enhances *OqxAB* transcription (Wan Nur Ismah et al., 2018). Furthermore, it has been demonstrated that mutations such as V57L in the *rpsJ* gene can contribute to resistance even in the absence of *ramR* mutations (Herrera et al., 2021). The tet protein also has several known tigecycline resistance mechanisms, including *tet(A)*, *tet(L)*, *tet(X)*, and *tet(M)* (Fiedler et al., 2016; Fan et al., 2024; Zou et al., 2024). Among them, mutations in the ribosome protection protein *tet(M)* can modify drug resistance by changing the binding site. Tetracycline mobile inactivating enzyme *tet(X)* and its variants can exist on a variety of mobile genetic elements, mediate the rapid spread of tigecycline resistance genes through horizontal transfer, and exist stably in drug-resistant strains at a very low adaptive cost, and significantly increase the level of resistance to tigecycline (Song et al., 2020; Hsieh et al., 2021).

The growing tigecycline resistance has significantly limited clinical treatment options for multidrug-resistant *K. pneumoniae*. Hence, it is crucial to examine TRKP isolates, particularly in regions like Southwest China, where data is lacking. This study aimed to evaluate the phenotypic characteristics, molecular prevalence and tigecycline resistance mechanism of TRKP isolates from southwest China. In brief, a drug susceptibility test was performed on clinically isolated *K. pneumoniae*, and the genetic relationship of TRKP isolates was studied using multi-site sequence typing (MLST) technique. At the same time, efflux pump inhibition assay was performed to verify efflux pump activity. Using PCR, DNA sequencing technology and reverse transcription PCR (RT-PCR), the determinants of drug resistance including tigecycline resistance genes, pump genes and their regulatory factors were studied.

2 Materials and methods

2.1 Identification of strains and drug susceptibility test

Between August 2021 and December 2023, we used the Vitek-2 system (Biomérieux, France) to isolate TRKP strains from southwest China. Through the MALDI-TOF mass spectrometry (Biomérieux, Craponne, France), all separate strains are recognized as *K. pneumoniae*. The minimum inhibitory concentration (MIC) was determined with cation-regulated Mueller Hinton broth (CAMHB), and *Escherichia coli* ATCC 25922 was used as the control strain. Since CLSI has not yet determined the breakpoint of tigecycline, this study referred to FDA's sensitivity guidelines for Enterobacteria (sensitivity $\leq 2\text{mg/L}$, intermediate 4mg/L , resistance $\geq 8\text{ mg/L}$) (Zheng et al., 2018). Following CLSI-2023 recommendations, the VITEK-2 system was used to carry out further susceptibility to antimicrobial investigation, and the outcomes were interpreted appropriately.

2.2 Identification of determinants of tigecycline resistance

As indicated in Table 1, PCR was performed using gene-specific primers to test TRKP clinical isolates for the tigecycline resistance

determinants *acrR*, *ramR*, *rpsJ*, *oqxR*, *tet(L)*, *tet(A)*, *tmexC* and *tet(X)*. In order to provide a reliable comparison basis after sequencing, so as to accurately identify and locate the mutation sites generated by our experiment, the sequences were compared to those of wild-type reference strains *E. coli* plasmid RP1 for *tet(A)* detection (GenBank accession number X00006)] and [*K. pneumoniae* MGH78578 (GenBank accession number CP000647) for other detection to show the mutations. A total of 35 cycles of PCR reaction conditions were as follows: predenaturation at 94°C for 5 minutes; denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 40 seconds; and finally, extension at 72°C for 5 minutes.

2.3 Efflux inhibition assay

Utilizing the efflux pump inhibitor (EPI) Phe-Arg- β -naphthylamide (PA β N, MedChemExpress), the efflux pump activity in isolates of *K. pneumoniae* resistant to tigecycline was investigated. The MIC of tigecycline both with and without PA β N at a concentration of 50 mg/l was determined using the broth microdilution technique. When EPIs are present, a quadruple or higher decline in the MIC is deemed to be evidence of efflux pump efficiency.

2.4 Quantitative reverse transcription PCR

Using qRT-PCR, the expression levels of the transcriptional regulator genes *ramA* and the efflux pump genes *acrB* and *oqxB* were evaluated. As directed by the manufacturer, total RNA from bacteria has been extracted using the RNeasy Pure Cell/Bacteria Kit (Tiangen, Beijing, China). The PrimeScript™ FAST RT Reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan) was subsequently utilized to synthesize cDNA. Each sample was analyzed in triplicate. Normalization of the target gene's mRNA expression was done using the housekeeping gene, *rpoB*. The relative expression level of tigecycline-sensitive bacteria ATCC 25922 was measured as a negative control. Cycle threshold (Ct) values were measured by the qRT-PCR program, and analysis of results was carried out using the 2- $\Delta\Delta$ Ct method.

2.5 Multilocus sequence typing

MLST has been used to analyze the genetic relationships of the strains. We download primers from PubMLST website (<http://www.pasteur.fr/recherche/genopole/PF8> MLST/Kpneumoniae HTML) for seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*), amplify and sequence the genes, and then analyze them using the MLST database. The analysis utilized the PubMLST site (<http://www.pasteur.fr/recherche/genopole/PF8> MLST/Kpneumoniae HTML), 7 housekeeping gene synthesis listed on the website of the primer. The genes were amplified and sequenced, with subsequent analysis performed using the MLST database (Veleba et al., 2012). Sequence types (STs) were classified into clonal complexes (CCs) using the eBURST algorithm.

Statistical methods GraphPad Prism software V. 9.5.0 (GraphPad Software Inc, San Diego, CA) was used to statistically analyze the correlation between anti-tigecycline resistance and *acrB*, *oqxB*, and *ramA* expression levels. The calculation of gene expression differences between the groups was based on the Mann–Whitney U test and a P value less than 0.05 was considered statistically significant.

3 Results

3.1 Clinical characteristics of TRKP isolates

Analysis of specimen sources showed that most TRKP isolates were derived from urine (30.0%) and respiratory secretions (43.4%),

TABLE 1 Clinical Characteristics of TRKP Isolates (n = 30).

Characteristics	No. (%)
Type	
Sputum	11(36.7)
Urine	9(30)
Bronchoalveolar lavage	2(6.7)
bile	4(13.3)
Drainage fluid	1(3.3)
Blood	1(3.3)
Other	2(6.7)
Department distribution	
Intensive Care Unit	10(33.3)
Urinary Surgery	4(13.3)
Hepatobiliary Surgery	4(13.3)
Rehabilitation department	3(10)
Neurology Department	2(6.7)
Gastrointestinal Surgery	2(6.7)
kidney internal medicine	2(6.7)
Oncology Department	1(3.3)
Orthopedics	1(3.3)
geriatric department	1(3.3)
Sex	
Male	20(66.7)
Female	10(33.3)
Age (mean $\pm$ SD)	68.83 $\pm$ 17.73
Use of tigecycline	6(20.0)
Death rate	11(37.7)
Day(s) of hospitalization	69.07 $\pm$ 14.53

TRKP, Tigecycline-resistant *K. pneumoniae*.

of which sputum accounted for 36.7% and bronchoalveolar lavage fluid accounted for 6.7% (see Table 1). Of these 30 isolates, almost a third of the samples were taken from intensive care units (ICU). The TRKP strain affected 20 cases (66.7%) in males and 10 cases (33.3%) in women, with a median age of 68 years. (Table 1) All clinical specimens have been identified as *K. pneumoniae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (bioMérieux, Marcy-l'Étoile, France).

3.2 Antibiotic resistance profile and Efflux pump inhibition assay in TRKP isolates

In this study, the tigecycline drug sensitivity and related characteristics of TRKP clinical isolates were detected (Table 2). Based on FDA guidelines, isolates with tigecycline MICs ≥ 8 $\mu\text{g/ml}$ were classified as resistant. Among the 30 isolates, 50% had MICs of 8 $\mu\text{g/ml}$, 30% had MICs of 16 $\mu\text{g/ml}$, and 20% had MICs of 32 $\mu\text{g/ml}$. All TRKP isolates were resistant to minocycline. These isolates were also co-resistant ciprofloxacin (25/30, 83.4%), levofloxacin (21/30, 70.0%), cefoperazone (19/30, 63.4%), cefepime (19/30, 63.4%), ceftriaxone (18/30, 60.0%), aztreonam (13/30, 43.3%), Ceftazidime (12/30, 40.0%), ceftazidime (11/30, 36.7%), gentamicin (5/30, 16.7%), and amikacin (4/30, 13.4%) were also co-resistant, suggesting that the majority of the strains were MDR. However, no colistin and meropenem resistant isolates were found. We conducted efflux pump inhibition experiments to investigate the mechanism of tigecycline resistance in *K. pneumoniae*. After exposure to efflux pump inhibitor (EPI) phenylalanine-arginine- β -naphthylamide (PA β N), 96.6% of 30 tigecycline-resistant strains (MIC ≥ 8 mg/L) recovered their sensitivity. Among the 30 tigecycline-resistant isolates (MIC ≥ 8 mg/L), one isolate exhibited a 32-fold reduction, 11 isolates had a 16-fold reduction, 15 showed an eightfold reduction, two isolates showed a fourfold reduction, and one isolate's tigecycline MIC remained unchanged With PA β N. The effects of PA β N on mic of tigecycline are shown in Table 2.

3.3 Identifying and sequencing determinants of tigecycline resistance in clinical isolates of TRKP.

To explore the mechanisms underlying tigecycline resistance in TRKP, we identified potential tigecycline resistance determinants through PCR and sequencing, specifically including *ramR*, *acrR*, *oqxR*, *tet(A)*, *tet(X)*, *tet(L)*, *tmexC*, and *rpsJ* (Table 2). Using published primers (Supplementary Table S1), *rpsJ* gene was detected in all isolates. *oqxR* and *acrR* were detected in 26 isolates (86.6%), *ramR* was detected in 25 isolates (83.3%), and *tet(A)* was detected in 19 isolates (63.3%). No strains carrying *tet(X)* and *tet(L)* were detected (Table 2). Sequence alignment diagrams containing protein mutations are shown in Supplementary Figures S1–S4.

In comparison with the standard WT strain MHG78578 (GenBank number CP000647), 22 isolates (22/30, 73.3%) showed nucleotide changes in *ramR*. Five isolates (5/22, 22.7%) had a

deletion of *ramR* gene, and 17 isolates (17/22, 77.3%) carried frameshift or substitution mutations in *ramR*, most of the substitutions exist in the DNA-binding domain. Among the more common *ramR* mutations are A19V substitution, K63M substitution, and I141T substitution (Table 1). Seven isolates produced truncated RamR proteins, including TR17 (181 amino acids [aa]), TR7 (121 aa), TR5 (110 aa), TR28 (98 aa), TR23 (42 aa), TR24 (40 aa) and TR19 (19 aa) (Figure 1).

The *tet(A)* variant is a major cause of tigecycline resistance: type 1 (n=17) and type 3 (n=2). Type 3 *tet(A)* showed a single amino acid difference from type 1 and a 28-bp nucleotide deletion.

The amino acid substitution involved in two *acrR* mutants (S215P, Y114F, V165I in TR6 and E200V in TR18) has not been previously reported. Of the three *oqxR* mutant isolates (E24R in TR1, C100Y in TR19, and 226–227 base deletion in TR29), E24R mutation in TR1 isolates did not lead to overexpression of *oqxR*. Recently, mutations in the *rpsJ* gene encoding the ribosomal protein S10 have been reported to be associated with tigecycline resistance in *Klebsiella pneumoniae*. Although *rpsJ* gene was present in all 30 TRKP isolates in our study, no *rpsJ* gene mutation was found. The *tmexC* gene was detected in 2 TRKP isolates. The ST1561 TmexCD-ToprJ-positive TRKP strain did not use tigecycline during treatment, and eventually the patient recovered and was discharged successfully. Resistance gene screening revealed the presence of type 1 *tet(A)* mutants in this strain, along with A2G mutations in *ramR*, showing expression of *rpsJ* and *oqxR* genes, but not *acrR*. In contrast, the ST661 strain was treated with tigecycline, but the patient sadly died. The strain expresses type 1 *tet(A)* mutant, *rpsJ*, *ramR* and *acrR*, but does not express *oqxR*. Additionally, no mobilized tigecycline resistance genes, such as *tet(X)*, were detected.

3.4 Pump and regulator gene expression of TRKP isolates.

We evaluated expression levels of efflux pump AcrAB-TolC, OqxAB, and transcriptional regulatory gene *ramA*. The qRT-PCR analysis showed that 22 of the 30 Tigecycline-resistant strains had overexpression of *acrB* gene (5.49- to 48.49- fold) and *ramA* gene (5.20–83.93 fold). However, in the OqxAB efflux pump pathway, only 4 strains overexpressed the *oqxR* gene (9.02- to 60.07- fold) (Figures 2A–C). TRKP collected was divided into three groups according to MIC value (MIC=8 $\mu\text{g/ml}$, MIC=16 $\mu\text{g/ml}$, MIC=32 $\mu\text{g/ml}$). Compared with the group with MIC of 8 $\mu\text{g/ml}$, *acrB* was statistically significant when MIC was 32 groups (p 0.0302). There were significant differences in *ramA* when MIC was 16 (p 0.0248) (Figure 2D).

3.5 Molecular Epidemiology based on MLST

Twenty-five different STs were observed in 30 TRKP isolates. ST307 was the dominant strain, accounting for 4 strains (11.8%), followed by ST15 type, accounting for 2 strains (6.7%). No novel ST types were detected in this study.

4 Discussion

The majority of TRKP strains in our hospital come from older adults and those in the ICU with catheters. Severe pneumonia caused by TRKP often results in extended hospital stays (3-370 days) and poor outcomes, with a 36.7% mortality rate. *K. pneumoniae* comes from a variety of specimens and can cause infections of the lungs, urinary tract, tissues, bile, and ducts. Antimicrobial susceptibility tests show that TRKP isolates exhibit high resistance to most antibiotics. The increased MIC of tigecycline may be linked to the use of other antibiotics, which are also expelled via the AcrAB-TolC pump (Roy et al., 2013). In this study, 80% of patients had no prior exposure to tigecycline, confirming that

resistance can occur without direct exposure. Efflux pump inhibitors (EPIs) down-regulate their expression by interfering with the combination of efflux pump-related proteins, blocking the energy supply effect, and inhibiting the substrate's passage through efflux pump channels [16]. To detect the presence of overexpression of efflux pumps in TRKP strains, we used efflux pump inhibitor (EPI) PA β N to evaluate efflux pump activity. In our study, 96.6% of TRKP strains showed a fourfold or greater reduction when PA β N was present. The MIC of tigecycline remained unchanged when PA β N was present in TR23. We believe that the following factors may be involved: First, changes in membrane permeability, such as mutations of ompK35 and ompK36 genes, may lead to decreased permeability of the outer

TABLE 2 TGC resistance determinants mutation(s) of TRKP isolates examined in the present study.

		TGC		Presence of TGC resistance determinants [mutation(s) occurring in nucleotide or protein sequence] ^a							
Isolate	TGC	+PA β N	ST	<i>ramR</i>	<i>acrR</i>	<i>oqxR</i>	<i>Tet(A)</i>	<i>tet(X)</i>	<i>tet(L)</i>	<i>tmexc</i>	<i>rpsJ</i>
TR1	8	1	6148	+	+	+(E24R)	–	–	–	–	+
TR2	8	1	15	+(A19V, K63M)	+	+	+(type 1)	–	–	–	+
TR3	8	2	15	+(A19V, K63M)	+	+	+(type 1)	–	–	–	+
TR4	8	1	65	–	+	+	–	–	–	–	+
TR5	8	0.5	1838	+	+	+	–	–	–	–	+
TR6	8	1	1308	–	+(S215P, Y114F, V165I)	–	–	–	–	–	+
TR7	8	2	4106	+(11nt Δ (365-375))	+	+	+(type 1)	–	–	–	+
TR8	8	1	45	+	+	+	+(type 1)	–	–	–	+
TR9	8	1	893	+(R121P)	+	+	+(type 3)	–	–	–	+
TR10	8	1	307	+	+	–	+(type 1)	–	–	–	+
TR11	8	1	307	+	+	–	+(type 1)	–	–	–	+
TR12	8	1	307	+(S157L)	+	+	–	–	–	–	+
TR13	8	0.5	1561	+(A2G)	–	+	+(type 1)	–	–	+	+
TR14	8	1	3096	+(stop194K)	+	+	+(type 1)	–	–	–	+
TR15	8	0.5	875	+10nt Δ (332-341))	+	+	–	–	–	–	+
TR16	16	1	23	+	+	+	–	–	–	–	+
TR17	16	1	700	+(E182stop)	+	+	+(type 1)	–	–	–	+
TR18	16	2	6092	+(A34V)	+(E200V)	+	+(type 1)	–	–	–	+
TR19	16	0.5	101	+(1nt Δ (57bp))	+	+(C100Y)	–	–	–	–	+
TR20	16	2	661	+	+	–	–	–	–	+	+
TR21	16	1	307	–	+	–	+(type 1)	–	–	–	+
TR22	16	1	3368	–	–	+	+(type 1)	–	–	–	+
TR23	16	16	11	+(8nt Δ (128-135bp))	–	–	+(type 1)	–	–	–	+
TR24	16	2	37	+(E41stop)	+	+	+(type 1)	–	–	–	+
TR25	32	2	485	+	+	+	+(type 1)	–	–	–	+
TR26	32	2	29	+(Y59H, I141T)	+	+	+(type 1)	–	–	–	+

(Continued)

TABLE 2 Continued

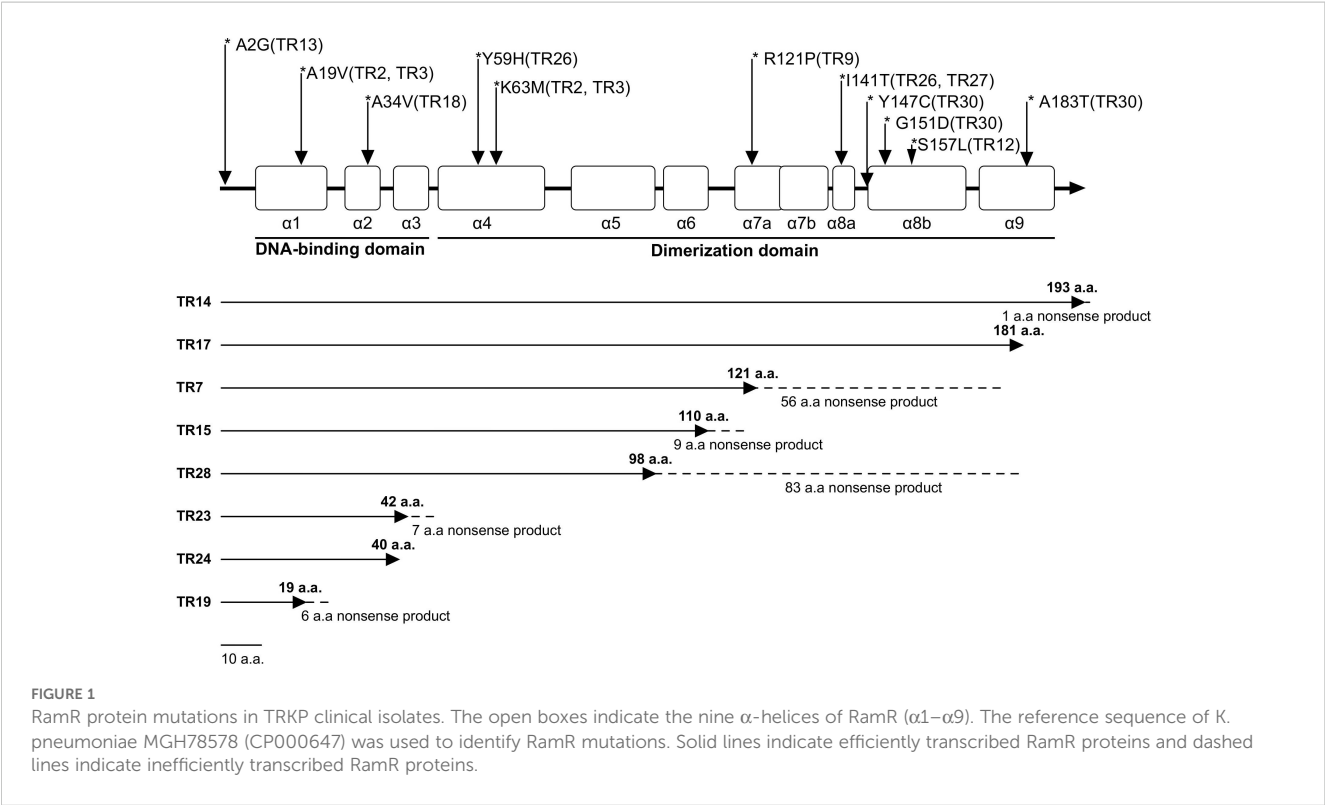
		TGC		Presence of TGC resistance determinants [mutation(s) occurring in nucleotide or protein sequence] ^a							
Isolate	TGC	+PAβN	ST	<i>ramR</i>	<i>acrR</i>	<i>oqxR</i>	<i>Tet(A)</i>	<i>tet(X)</i>	<i>tet(L)</i>	<i>tmexc</i>	<i>rpsJ</i>
TR27	32	4	65	+(I141T)	+	+	+(type 1)	–	–	–	+
TR28	32	2	25	+(C292ins-frameshift mutation, E182*)	+	+	–	–	–	–	+
TR29	32	2	96	–	–	+(2nt Δ (226-227))	–	–	–	–	+
TR30	32	4	5387	+(Y147C,G151D,A183T)	+	+	+(type 3)	–	–	–	+

^aGenetic determinants of resistance were detected by performing PCR with gene-specific primers. Mutations were identified by comparison with wild-type reference sequences [Klebsiella pneumoniae MGH78578 (CP000647) for *ramR*, *acrR*, *oqxR* and *rpsJ*, Escherichia coli for *tet(A)* (X00006)]. +, presence of target gene and no change in the nucleotide or amino acid sequence; –, absence of target gene; Δ, deletion; bp, base pair.

membrane to tigecycline; Second, in the presence of PAβN, other efflux pumps may still expel tigecycline. Finally, the metabolic status of bacteria may also affect their susceptibility to drugs. We plan to further explore these resistance mechanisms through whole genome sequencing. However, most of the existing efflux pump inhibitors have obvious toxicity, and how to develop clinical drugs with high specificity, low toxicity, and high safety remains to be further explored.

Tet (A) belongs to the MFS efflux pump family with mutations that allow tigecycline to accumulate within bacterial cells leading to resistance (Linkevicius et al., 2016). In 2017, Chiu et al. first discovered that type 1 *tet(A)* raised the MIC of tigecycline by a factor of 8. It was also demonstrated that in the case of *tet(A)* mutation, loss of *RamR* protein has a synergistic effect on

tigecycline resistance in *K. pneumoniae* (Chiu et al., 2017). Five years later, Peng et al. demonstrated through cloning experiments that type 3 *tet (A)* could raise the MIC of tigecycline fourfold (Peng et al., 2022a). In our experiments, we found that almost all isolates carried *tet (A)* mutations or *ramR* mutations. No *ramR* was detected in five of the isolates, suggesting that their *ramR* gene may have been truncated or deleted, similar to the fully *ramR* deletion mutant of *Klebsiella pneumoniae* strain KPBj1 M3 Lev (Bialek-Davenet et al., 2013). Furthermore, the deletion and insertion of various fragments among the seven isolates contributed to the premature appearance of the stop codon. This may result in the loss of the α8-α9 region, disrupt dimerization, and ultimately lead to a loss of function (Yamasaki et al., 2013). Fortunately, A large study has shown that although *ramR* mutants can enhance bacterial



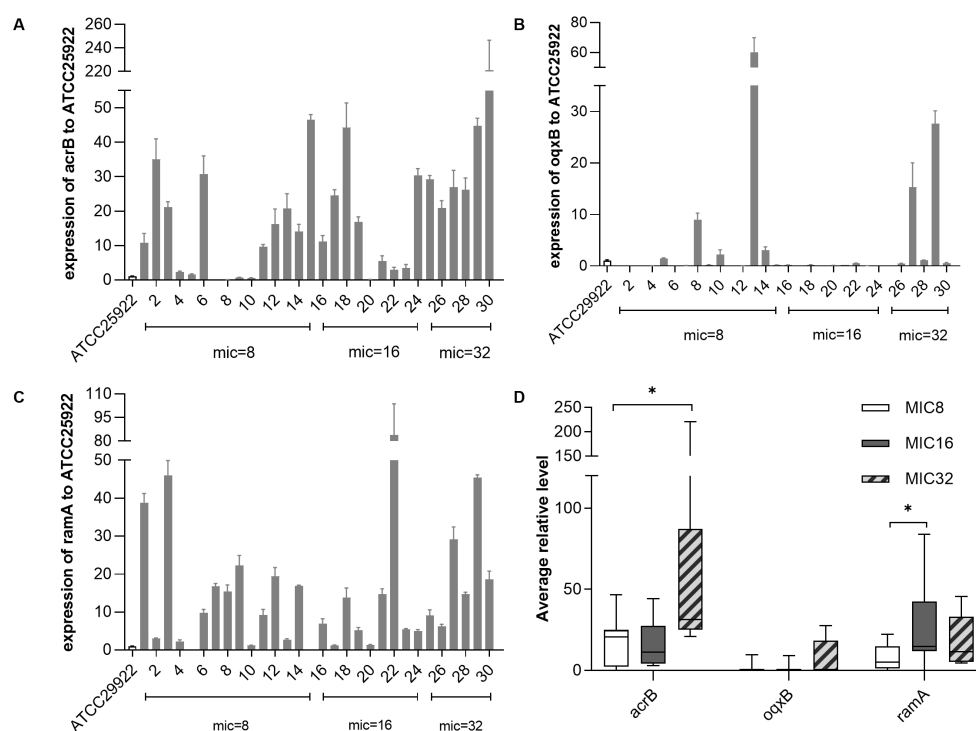


FIGURE 2

The expression levels of resistance-nodulation-cell division (RND) efflux pump gene and *ramA* gene in *Klebsiella pneumoniae*. The expression levels of resistance-nodulation-cell division (RND) efflux pump gene and *ramA* gene in *K. pneumoniae* were determined by qPCR and the target gene expression levels were divided into three groups according to the MIC value of tigecycline (=8 mg/L, = 16mg/L, = 32mg/L) for comparison, with tigecycline-susceptible *Escherichia coli* ATCC 25922 as control (expression = 1). (A) Relative expression (RE) level of *acrB* in TRKP strains; (B) RE level of *oqxR* in TRKP strains; (C) RE level of *ramA* in TRKP strains; (D) Average RE of *acrB*, *oqxR*, and *ramA* in TRKP isolates treated with different tigecycline MIC values. The bars represent the average value and the error bars represent the standard error of the mean value. Data were analyzed by Mann–Whitney U test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

resistance, they also induce an enhanced immune response by modulating the structure of lipid A, thereby reducing the pathogen's ability to kill in organs and blood (Yu et al., 2024).

Point mutations in the local suppressor *oqxR* have been shown to cause the OqxAB efflux pump to become overactive, giving the isolates increased virulence and multidrug-resistant characteristics (Bialek-Davenet et al., 2015). Previous studies have shown that amino acid substitution mutations such as V102G and V130A confer resistance to tigecycline (Bialek-Davenet et al., 2015; Chiu et al., 2017). This paper identifies for the first time two additional amino acid substitutions (E24R and C100Y) and frameshift deletions involving amino acids 226 to 227, which may negatively impact function. Therefore, the effect of these mutations on drug resistance requires further investigation. The recent discovery of the novel tetracycline-inactivating enzyme *tet(X)* homologs and the efflux pump gene clusters *Tmexd-toprJ* has been linked to high levels of tigecycline resistance. These resistance mechanisms can be horizontally transferred via mobile elements like plasmids, spreading to humans, animals, and the environment, and posing a significant public health threat (Guo et al., 2024; Pan et al., 2024). A report identified 237 bacterial strains worldwide carrying the *tmexCD-toprJ* gene, with 92.83% originating from China (Dong et al., 2022). These strains represent 50 unique sequence types. Our study marks the first identification of two novel *tmexCD-toprJ*-

positive strains, ST661 and ST1561, offering fresh insights into microbial research and underscoring the significance of bacterial diversity alongside its vast research potential. *Tmexcd*-positive strains were previously found mainly on chromosomes, but the discovery of plasmids in the past five years suggests a possible transmission mechanism. Despite attempts to perform conjugation experiments to determine whether *tmexC* is located on the plasmid and its ability to transfer, they were unsuccessful, suggesting that they may indeed be located on chromosomes. To further test this hypothesis, whole genome sequencing is planned to confirm the specific location and function of *tmexCD-toprJ*. In addition, patients infected with ST1561 were observed to survive, while those infected with ST661 died, a difference that may reflect significant differences in the pathogenicity and virulence of the strains. *rpsJ* mutations linked to tigecycline resistance are generally found in the amino acids located between positions 53 and 60 of the S10 ribosomal protein (Beabout et al., 2015). Fortunately, we did not find any *rpsJ* mutations related to tigecycline in the strains, nor did we detect any cases of *tet(X)* carrying.

Both AcrAB and OqxAB efflux pumps are common resistance mechanisms in enterobacteria (Liu et al., 2018). Research shows that OqxAB overexpression can lead to resistance to several antimicrobials, including chloramphenicol, quinolones, furantoin, and tigecycline (Li et al., 2019). In our study, *acrB* expression

increased with varying MIC levels, while *oqx*B was overexpressed in only 4 bacterial strains. This aligns with findings by Perez et al., showing that while OqxAB is widely distributed in *K. pneumoniae*, it is not always overexpressed (Perez et al., 2013). It is important to note that the *ram*R gene was absent from the highly resistant strain TR4 (MIC 8 µg/ml), and efflux pump inhibition tests demonstrated that the resistance mechanism was linked to the efflux pump even though *acr*B expression was only 2.32 times higher. Furthermore, no known resistance determinants were discovered, indicating that resistance may emerge through other pathways such as KpgABC or MacAB-TolC efflux pumps. Mutation of *ram*R, as an inhibitor of *ram*A, leads to up-regulation of *ram*A expression, which in turn increases the expression of *acr*AB or *oqx*AB efflux pump. In this study, three isolates (TR10, TR16, TR25) showed elevated *ram*A expression without any *ram*R mutations. This may be linked to mutations in the RamR recognition sites (PI and PII promoters) or Lon protease mutations (Rosenblum et al., 2011; Ricci et al., 2014), requiring further investigation to clarify this finding. Although it has been established that efflux mechanisms generally only lead to low levels of resistance to tigecycline, we speculate that multiple mechanisms may be at work at the same time, with possible synergistic effects. Therefore, more in-depth research is urgently needed to explore the interrelationships and effects of these mechanisms.

Additionally, 30 *K. pneumoniae* isolates were classified into 25 distinct ST types, reflecting the significant genetic diversity of TRKP. Previous research has indicated that most TRKP strains are resistant to multiple antibiotics and carry virulence factors, heightening the risk of resistance and virulence gene transfer. Monitoring the spread of these clones is essential to prevent their emergence as clinical pathogens (Peng et al., 2022a).

This study's limitation is its single-center scope, which may limit representativeness. Additionally, only certain resistance genes were screened, leaving other mechanisms unexplored. Future efforts will expand sample size through multi-center studies and broaden genetic screening to better understand resistance mechanisms and enhance the study's clinical relevance. In conclusion, *acr*B overexpression and *tet*(A) mutations are key contributors to tigecycline resistance in *K. pneumoniae* in southwest China.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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Ethics statement

The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (2024-373-01).

Author contributions

YL: Writing – original draft, Writing – review & editing, Formal Analysis. ST: Writing – review & editing. QH: Supervision, Writing – review & editing. PX: Supervision, Writing – review & editing. TS: Supervision, Writing – review & editing. YS: Supervision, Writing – review & editing. YX: Writing – review & editing.

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Supplementary material

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Involvement of the *bla*_{CTX-M-3} gene in emergence of a peculiar resistance phenotype in *Klebsiella pneumoniae*

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Introduction: This study aimed to investigate the mechanism underlying a peculiar resistance phenotype in *Klebsiella pneumoniae*, characterized by reduced susceptibility to cefepime compared to ceftazidime.

Methods: Antimicrobial susceptibility testing, plasmid conjugation experiments, whole-genome sequencing, and bioinformatic analyses were employed to characterize the resistance phenotype and identify genetic determinants.

Results: A total of 20 *K. pneumoniae* strains exhibiting peculiar resistance phenotypes were collected and analyzed. Ten distinct sequence types (STs) were identified, including ST25 (4/20), ST967 (3/20), ST65 (2/20), ST133 (2/20), ST48 (2/20), ST353 (1/20), ST628 (1/20), ST753 (1/20), ST792 (1/20), and ST254 (1/20). All strains were resistant to FEP (MIC₅₀ = 128 µg/mL) but not to CAZ (MIC₅₀ = 8 µg/mL). This resistance was primarily attributed to the presence of the *bla*_{CTX-M-3} (14/20) and *bla*_{OXA-10} (3/20). Conjugation experiments demonstrated that 5 out of 14 *bla*_{CTX-M-3}-positive *K. pneumoniae* strains successfully acquired transconjugants, which exhibited the same peculiar resistance phenotype. PCR analysis confirmed that the conjugates contained the IncFII plasmid. To further elucidate the genetic basis of the resistance phenotype, whole-genome long-read sequencing was performed on three *bla*_{CTX-M-3}-positive *K. pneumoniae* strains. The sequencing results confirmed that *bla*_{CTX-M-3} was located on the IncFII plasmid, and analysis of its genetic environment revealed a frequent association with mobile genetic elements such as IS26, ISEcp1, and Tn3.

Discussion: The primary driver of this phenotype in *K. pneumoniae* is the presence of the IncFII plasmid carrying *bla*_{CTX-M-3}, which contrasts with the resistance mechanisms often reported in *Pseudomonas aeruginosa* exhibiting similar phenotypes. This study emphasizes the critical role of plasmid-mediated resistance in the spread of multidrug resistance in *K. pneumoniae* and provides insights into strategies for combating resistance in these pathogens.

KEYWORDS

CTX-M-3, *Klebsiella pneumoniae*, peculiar resistance phenotype, cefepime, ceftazidime

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a significant Gram-negative bacillus (GNB) responsible for nosocomial infections, including pneumonia, urinary tract infection, and bloodstream infections (Munoz-Price et al., 2013). The rise in multidrug-resistant strains of *K. pneumoniae* has heightened concerns in clinical environments, complicating treatment protocols and contributing to increased morbidity and mortality (Liu et al., 2022). Cephalosporins, particularly third and fourth-generation agents, are widely used for treating *K. pneumoniae* infections due to their broad-spectrum efficacy. However, certain *K. pneumoniae* strains have demonstrated a peculiar resistance phenotype, showing reduced susceptibility to the fourth-generation cephalosporin cefepime (FEP) compared to the third-generation cephalosporin ceftazidime (CAZ). This phenotype is consistently referred to as the “peculiar resistance phenotype” throughout the article. Importantly, the clinical significance of this resistance phenotype seems to be largely overlooked. This atypical resistance pattern poses a clinical challenge, as FEP is generally considered more potent than CAZ against resistant strains.

FEP exhibits potent antibacterial activity against GNB, including *K. pneumoniae* and *Escherichia coli* from the *Enterobacteriaceae* family, as well as non-fermentative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) (Okamoto et al., 1994; Nguyen et al., 2014; Rodriguez-Bano et al., 2018). Notably, a review of the literature indicates that research on this peculiar resistance phenotype primarily focuses on *P. aeruginosa*, with limited studies addressing other bacterial species. Mechanisms such as the overexpression of the *mexXY*-OprM efflux system and the emergence of various β -lactamases, including *bla*_{OXA-1}, *bla*_{OXA-10}, *bla*_{OXA-31}, *bla*_{OXA-35}, and *bla*_{PSE-1}, have been identified as significant contributors (Aubert et al., 2001; Hocquet et al., 2006; Campo Esquisabel et al., 2011). These mechanisms enable the bacteria to evade the β -lactam antibiotics, leading to treatment failures. Although *K. pneumoniae* is one of the common pathogenic GNB, reports detailing the mechanisms underlying this peculiar resistant phenotype remain limited. This raises the question of whether there are differences in resistance mechanisms between *K. pneumoniae* and *P. aeruginosa*. This gap in the literature underscores a critical need for further investigation into the genetic and biochemical pathways involved. Understanding these mechanisms is crucial for developing effective therapeutic strategies to combat the rising threat of antibiotic resistance in *K. pneumoniae*.

Materials and methods

Bacterial strains

Between January 1, 2023, and June 30, 2023, peculiar resistance phenotypes of *K. pneumoniae* strains were isolated from patient specimens at the Fifth Affiliated Hospital of Southern Medical University, China. To ensure the integrity of the subsequent

research, a total of 20 unique *K. pneumoniae* were selected after removing duplicates. All strains were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS; bioMérieux, France).

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) values for FEP, CAZ, amikacin (AMK), amoxicillin-clavulanic acid (AMC), ertapenem (ETP), cotrimoxazole (SXT), piperacillin-tazobactam (TZP), tigecycline (TGC), cefuroxime (CXM), cefuroxime axetil (CXA), ceftriaxone (CRO), ceftazidime (FOX), imipenem (IPM), and levofloxacin (LVX) were performed using VITEK-2 Compact equipment. To precisely determine the MICs of CAZ and FEP, broth microdilution assays were performed in sterile 96-well plates. Twofold serial dilutions of antimicrobial agents were prepared in Mueller-Hinton broth (MHB) with the following concentration ranges: FEP from 1,024 to 0.0625 $\mu\text{g/mL}$ and CAZ from 16 to 0.0625 $\mu\text{g/mL}$. Bacterial suspensions were standardized to 0.5 McFarland units ($\sim 1 \times 10^8$ CFU/mL) and diluted 1:100 in MHB prior to inoculation. Each plate included growth controls (broth + inoculum without antibiotics) and sterility controls (broth only). Plates were incubated aerobically at 35°C for 18–20 hr before visual MIC determination. All procedures and result interpretations followed the Clinical and Laboratory Standards Institute (CLSI) guidelines, CLSI M100-2024.

Plasmid conjugation experiments

The transferability of plasmids carrying resistance-related genes was determined by the conjugation experiments using *K. pneumoniae* exhibiting peculiar resistance phenotype as donors and rifampin-resistant *E. coli* C600 as a recipient. Transconjugants were selected on Luria-Bertani agar plates supplemented with rifampin (100 $\mu\text{g/mL}$) and FEP (4 $\mu\text{g/mL}$). PCR sequencing (refer to Supplementary Table S1 for the relevant primer sequences) was employed to verify the presence of the *bla*_{CTX-M-3} gene in the transconjugants. Subsequently, antimicrobial susceptibility testing was conducted to confirm the antimicrobial resistance profiles of these transconjugants.

Whole-genome sequencing and bioinformatics analysis

Genomic DNA from *K. pneumoniae* isolates exhibiting peculiar resistance phenotype was extracted using a bacterial genomic DNA extraction kit (Tiangen, Beijing, China) and subsequently sequenced on the Illumina NovaSeq 6000 platform. The original clean offline sequences were assembled with SPAdes 3.13.1 and annotated using Prokka 1.14.5 (Bankevich et al., 2012; Seemann, 2014). Sequence types (ST) were determined through multilocus sequence typing (MLST 2.18.0). The resistance genes and plasmid

replicon types of the isolates were analyzed using the ResFinder 4.1 and PlasmidFinder 2.1 web tools from the Center for Genomic Epidemiology (CGE), applying default thresholds of a minimum identity percentage of 90% and a minimum coverage percentage of 60% (Bortolaia et al., 2020). Five representative *K. pneumoniae* isolates exhibiting peculiar resistance phenotype were selected for sequencing on the Oxford Nanopore platform. A mixed genome assembly of long and short reads was conducted using Unicycler 0.4.8 (Wick et al., 2017). The various plasmid backbones carrying associated resistance genes were visualized through BRIG analysis, while the genetic context of different related resistance genes was illustrated using Easyfig (Alikhan et al., 2011; Sullivan et al., 2011). Additional genomic sequences of *K. pneumoniae* carrying *bla*_{CTX-M-3} gene were retrieved from the NCBI database and used for phylogenetic analysis alongside the strains sequenced in this study. Core-genome SNPs were extracted using Snippy v4.6.0 (<https://github.com/tseemann/snippy>). IQ-TREE2 v2.3.6 (<https://github.com/iqtree/iqtree2>) was then used to construct the phylogenetic tree with the TVM+F+ASC+R5 model.

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited in the DDBJ/ENA/GenBank under the accession IDs: JBJFKS000000000 to JBJFLL000000000. This range represents a collection of 20 genome sequences, each assigned a unique accession number. The individual genome data can be accessed through the respective accession numbers. The complete nucleotide sequences of plasmids pIncFII+FIB_OXA10-217-63, pIncFII_CTXM3_218-74, pIncFII_CTXM3_220-59, and pIncFII_CTXM3_221-24 have been uploaded to the NCBI GenBank database with the following accession numbers CP173724, CP173730, CP173733, and CP173739, respectively.

Results

Information on *K. pneumoniae* isolates with peculiar resistance phenotype

A total of 20 *K. pneumoniae* strains with peculiar resistance phenotype were collected and analyzed. Ten different sequence types (ST) were identified (Table 1). The most frequently detected ST was ST25, accounting for 20% (4/20), followed by ST967 at 15% (3/20). The sequence types ST65, ST133, and ST48 each represented 10% (2/20) of the isolates, while ST353, ST628, ST753, ST792, and ST254 were represented by a single strain. Two strains could not be assigned to any known ST. All strains exhibited resistance to FEP, with MIC values ranging from 32 to 512 µg/mL and a MIC₅₀ of 128 µg/mL. In contrast, none of the strains were resistant to CAZ, with MIC values ranging from 2 to 8 µg/mL and a MIC₅₀ of 8 µg/mL. Sensitivity to the carbapenems IPM, ETP and the aminoglycoside AMK was uniformly observed across all strains. The sensitivity rates for FOX and the β-lactam/

β-lactamase inhibitor combination TZP were 95% and 85%, respectively. In contrast, sensitivity rates for AMC and SXT were both 40%, while the sensitivity rate for the quinolone drug LVX was notably low at 5%. All strains demonstrated resistance to CRO, CXM, and CXA (Supplementary Table S3).

*bla*_{CTX-M-3} or *bla*_{OXA-10} contribute to the development of *K. pneumoniae* isolates with peculiar resistance phenotype

The *bla*_{CTX-M-3} gene was detected in 14 isolates, while *bla*_{CTX-M-14} was identified in 6 isolates and *bla*_{OXA-10} in 3 isolates (Table 1). The *bla*_{TEM-1} gene was identified in 11 isolates, all of which co-harbored *bla*_{CTX-M-3}. Transconjugants were successfully obtained from ten isolates through plasmid conjugation experiments. PCR analysis confirmed the presence of *bla*_{CTX-M-3} gene in five transconjugants, *bla*_{OXA-10} in two, and *bla*_{CTX-M-14} in three. These transconjugants displayed distinct resistance phenotypes (Supplementary Table S3). The MIC of FEP in transconjugants carrying *bla*_{CTX-M-3} and *bla*_{OXA-10} was 16 to 32 times higher than that of CAZ, whereas in transconjugants carrying *bla*_{CTX-M-14} the FEP MIC was only 1 to 2 times higher. By integrating the susceptibility profiles of the donor strain and the recipient strain C600, it was demonstrated that *bla*_{CTX-M-3} and *bla*_{OXA-10} contribute to the development of this peculiar resistance phenotype in *K. pneumoniae*. Additionally, nine isolates were found to harbor the *arr-3* gene, which mediates resistance to RIF and was identified as the primary factor causing the failure of the plasmid conjugation experiments. *bla*_{CTX-M-3}-positive isolates consistently carried multiple resistance genes, with the total number ranging from 7 to 22. Notably, most strains harbored more than 15 resistance genes. These genes conferred resistance to multiple drug classes, including β-lactams, quinolones, tetracyclines, aminoglycosides, and macrolides.

The location of *bla*_{CTX-M-3} and *bla*_{OXA-10} gene

The IncFII plasmid replicon was detected in all isolates, while the IncFIB plasmid replicon was identified in 15 of 20 isolates (75%). Additional plasmid replicons included IncI1 (3/20, 15%), IncHI1B (2/20, 10%), and IncR (2/20, 10%). The PCR test of the conjugate confirmed that both the *bla*_{CTX-M-3} and *bla*_{OXA-10} genes were carried by the IncFII plasmid (Supplementary Table S2). To further investigate the genomic context, five representative strains (217-63, 218-74, 219-96, 220-59, and 221-24) underwent third generation long-read sequencing. The analysis revealed that the *bla*_{CTX-M-3} gene in strains 219-96, 220-59, and 221-24, as well as the *bla*_{OXA-10} gene in strain 217-63, were all located on the IncFII plasmid. These findings corroborate the results of the conjugation experiments (Table 2).

Using the plasmid sequence identified in this study as references, circular plasmid maps were constructed using BRIG

TABLE 1 The genomic characteristics of *K. pneumoniae* isolates with a peculiar resistance phenotype.

Isolate	ST ^a	Specimen Type ^b	Resistance Genes ^c		Plasmid Replicon-types ^d	Plasmid Conjugation Experiments ^e
			Key Resistance Gene(s)	Other Resistance Genes		
217-59	–	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-61} , <i>oqx</i> A6, <i>oqx</i> B11, <i>tet</i> (A), <i>flo</i> R, <i>mph</i> (A), <i>aad</i> A16, <i>dfr</i> A27, <i>aac</i> (6')-Ib-D181Y, <i>qnr</i> B91, <i>qnr</i> S1, <i>sul</i> 1, <i>fos</i> A, <i>arr</i> -3	IncFII	Failure
217-63	25	sp	<i>bla</i> _{OXA-10} , <i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-110} , <i>oqx</i> B17, <i>oqx</i> A7, <i>qnr</i> S1, <i>bla</i> _{LAP-2} , <i>flo</i> R, <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>cml</i> A5, <i>aad</i> A1, <i>dfr</i> A14, <i>sul</i> 2, <i>fos</i> A6	IncFIB (2), IncI1, IncFII	Success
217-89	48	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-172} , <i>mph</i> (A), <i>sul</i> 1, <i>aad</i> A2, <i>dfr</i> A12, <i>fos</i> A, <i>qnr</i> S1, <i>aph</i> (3')-Ia	IncFII (2), IncFIB	Success
218-12	48	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-172} , <i>fos</i> A, <i>dfr</i> A12, <i>aad</i> A2, <i>sul</i> 1, <i>mph</i> (A), <i>qnr</i> S1, <i>aph</i> (3')-Ia	IncFII (2), IncFIB	Success
218-13	25	sp	<i>bla</i> _{OXA-10} , <i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-110} , <i>oqx</i> B17, <i>oqx</i> A7, <i>qnr</i> S1, <i>bla</i> _{LAP-2} , <i>flo</i> R, <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>cml</i> A5, <i>aad</i> A1, <i>dfr</i> A14, <i>sul</i> 2, <i>fos</i> A6	IncFIB (2), IncI1, IncFII	Success
218-19	25	cv	<i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-110} , <i>oqx</i> B17, <i>oqx</i> A7, <i>qnr</i> S1, <i>bla</i> _{LAP-2} , <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>tet</i> (A), <i>flo</i> R, <i>sul</i> 2, <i>fos</i> A6	IncFIB (2), IncI1, IncFII	Success
218-42	628	wd	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-110} , <i>oqx</i> A8, <i>oqx</i> B13, <i>tet</i> (A), <i>flo</i> R, <i>mph</i> (A), <i>aac</i> (6')-Ib-D181Y, <i>dfr</i> A27, <i>aad</i> A16, <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul</i> 2, <i>qnr</i> B91, <i>fos</i> A6, <i>qnr</i> S1, <i>aac</i> (3)-IId, <i>aph</i> (3')-Ia, <i>sul</i> 1, <i>arr</i> -3	IncFII	Failure
218-74	65	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{SHV-11} , <i>tet</i> (A), <i>flo</i> R, <i>sul</i> 2, <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>qnr</i> S1, <i>fos</i> A6, <i>oqx</i> B6, <i>oqx</i> A	IncFII, IncHI1B, IncFIB	Success
219-10	25	ab	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-110} , <i>flo</i> R, <i>tet</i> (A), <i>mef</i> (B), <i>sul</i> 3, <i>aad</i> A1, <i>cml</i> A1, <i>aad</i> A2, <i>dfr</i> A12, <i>aac</i> (6')-Ib-D181Y, <i>dfr</i> A27, <i>aad</i> A16, <i>sul</i> 1, <i>mph</i> (A), <i>sul</i> 2, <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>aac</i> (3)-IId, <i>qnr</i> S1, <i>oqx</i> A7, <i>oqx</i> B17, <i>aph</i> (3')-Ia, <i>fos</i> A6, <i>arr</i> -3	IncFIB, IncFII (2)	Failure
219-51	2154	ps	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-187} , <i>mph</i> (A), <i>sul</i> 1, <i>qnr</i> B91, <i>sul</i> 1, <i>aad</i> A16, <i>dfr</i> A27, <i>aac</i> (6')-Ib-D181Y, <i>tet</i> (A), <i>flo</i> R, <i>sul</i> 2, <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>qnr</i> S1, <i>aac</i> (3)-IId, <i>aph</i> (3')-Ia, <i>fos</i> A6, <i>oqx</i> A9, <i>oqx</i> B18, <i>arr</i> -3	IncFII	Failure
219-96	133	sp	<i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-75} , <i>oqx</i> B4, <i>oqx</i> A10, <i>fos</i> A6, <i>qnr</i> S1	IncFIB, IncFII (2)	Success
220-4	753	sp	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{OXA-10}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-110} , <i>fos</i> A, <i>mph</i> (A), <i>sul</i> 1, <i>qnr</i> B91, <i>sul</i> 1, <i>aad</i> A16, <i>dfr</i> A27, <i>aac</i> (6')-Ib-D181Y, <i>bla</i> _{LAP-2} , <i>qnr</i> S1, <i>flo</i> R, <i>tet</i> (A), <i>dfr</i> A14, <i>aad</i> A1, <i>cml</i> A5, <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul</i> 2, <i>oqx</i> A11, <i>oqx</i> B20, <i>aph</i> (3')-Ia, <i>arr</i> -3	IncFII (2), IncFIB	Failure
220-59	792	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-187} , <i>oqx</i> B19, <i>oqx</i> A9, <i>fos</i> A, <i>qnr</i> S1	IncFII	Success
220-61	133	sp	<i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-75} , <i>fos</i> A6, <i>oqx</i> A10, <i>oqx</i> B4	IncR, IncFII	Success
220-98	65	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{SHV-11} , <i>flo</i> R, <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul</i> 2, <i>qnr</i> S1, <i>fos</i> A6, <i>oqx</i> B6, <i>oqx</i> A	IncFII, IncHI1B, IncFIB	Success
221-5	–	cv	<i>bla</i> _{CTX-M-14}	<i>bla</i> _{SHV-75} , <i>oqx</i> B4, <i>oqx</i> A10, <i>tet</i> (A), <i>dfr</i> A1, <i>qnr</i> S1, <i>bla</i> _{LAP-2} , <i>tet</i> (D), <i>fos</i> A6	IncFII, IncFIB	Failure
221-22	967	bl	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-27} , <i>fos</i> A, <i>oqx</i> B21, <i>oqx</i> A6, <i>mph</i> (A), <i>sul</i> 1, <i>qnr</i> B91, <i>sul</i> 1, <i>aad</i> A16, <i>dfr</i> A27, <i>aac</i> (6')-Ib-D181Y, <i>flo</i> R, <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul</i> 2, <i>qnr</i> S1, <i>aac</i> (3)-IId, <i>aph</i> (3')-Ia, <i>arr</i> -3	IncFII (2), IncFIB	Failure
221-24	967	cv	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-27} , <i>fos</i> A, <i>oqx</i> A6, <i>oqx</i> B21, <i>mph</i> (A), <i>sul</i> 1(2), <i>qnr</i> B91, <i>aad</i> A16, <i>dfr</i> A27, <i>aac</i> (6')-Ib-D181Y, <i>flo</i> R, <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul</i> 2, <i>qnr</i> S1, <i>aac</i> (3)-IId, <i>aph</i> (3')-Ia, <i>arr</i> -3	IncFII (2), IncFIB	Failure

(Continued)

TABLE 1 Continued

Isolate	ST ^a	Specimen Type ^b	Resistance Genes ^c		Plasmid Replicon-types ^d	Plasmid Conjugation Experiments ^e
			Key Resistance Gene(s)	Other Resistance Genes		
221-29	353	wd	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-187} , <i>mph</i> (A), <i>qnrB91</i> , <i>sul1</i> (2), <i>aadA16</i> , <i>dfrA27</i> , <i>aac</i> (6')-Ib-D181Y, <i>floR</i> , <i>tet</i> (A), <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul2</i> , <i>aac</i> (3)-IId, <i>qnrS1</i> , <i>aph</i> (3')-Ia, <i>fosA6</i> , <i>oqx</i> A10, <i>oqx</i> B20, <i>arr-3</i>	IncFII, IncFIB	Failure
221-35	967	sp	<i>bla</i> _{CTX-M-3}	<i>bla</i> _{SHV-27} , <i>oqx</i> B21, <i>oqx</i> A6, <i>fosA</i> , <i>mph</i> (A), <i>sul1</i> , <i>aadA16</i> , <i>dfrA27</i> , <i>aac</i> (6')-Ib-D181Y, <i>tet</i> (A), <i>floR</i> , <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>sul2</i> , <i>qnrS1</i> , <i>aac</i> (3)-IId, <i>aph</i> (3')-Ia, <i>arr-3</i>	IncFIB (2), IncFII (2), IncR	Failure

a) “-” indicates that the ST type remains unidentified.

b) Specimen types: sp (sputum), cv (clean-catch midstream urine), wd (wound), ab (abscess), ps (pleural fluid), bl (blood).

c) Key Resistance Genes represent β-lactamases associated with the peculiar resistance phenotype.

d) The plasmid replicons identified in the strain are listed, with the number in parentheses indicating the count of replicons; data for the COL plasmid is not presented.

e) “Success” is defined as the successful acquisition of the transconjugant, while “failure” indicates that the transconjugant was not obtained. The primary reason for “failure” is the presence of the *arr-3* gene in the donor, which confers resistance to RIF.

(Figure 1). This analysis revealed three distinct plasmid backbones carrying the *bla*_{CTX-M-3} gene, each characterized by unique combinations of resistance genes within their variable regions (Figure 1B–D). In contrast, the plasmid backbone carrying the *bla*_{OXA-10} was identical across isolates, with a largely consistent set of associated resistance genes (Figure 1A).

The fusion plasmid in isolate 217-63, containing both the IncFII and IncFIB elements and carrying the *bla*_{OXA-10}, was designated plncFII+FIB_OXA10-217-63. This plasmid had a total length of 261,642 bp and carried ten additional resistance genes: *bla*_{LAP-2}, *sul2*, *aph*(3'')-Ib, *aph*(6)-Id, *tet*(A), *floR*, *cmlA5*, *aadA1*, *dfrA14*, and *qnrS1* (Figure 1A). Comparative BRIG analysis of other *bla*_{OXA-10}-positive isolates revealed that isolates 218-13 and 220-4 shared nearly identical plasmid backbones and resistance gene profiles with pIncFII+FIB_OXA10-217-63, suggesting a conserved genetic architecture among these plasmids.

The IncFII plasmid in isolate 218-74, carrying *bla*_{CTX-M-3}, was designated as plncFII_CTXM3_218-74. This plasmid spanned 119,574 bp and harbored additional resistance genes, including

sul2, *aph*(3'')-Ib, *aph*(6)-Id, *tet*(A), *floR*, and *qnrS1* (Figure 1B). In isolate 220-59, the IncFII plasmid carrying *bla*_{CTX-M-3} was named plncFII_CTXM3_220-59, with a total length of 71,104 bp. This plasmid also contained *bla*_{TEM-1} and *qnrS1* (Figure 1C). Similarly, the IncFII plasmid in isolate 221-24, referred to as plncFII_CTXM3_221-24, had a total length of 88,594 bp and encoded 16 additional resistance genes, including *bla*_{TEM-1}, *sul2*, *aph*(3'')-Ib, *aph*(6)-Id, *aph*(3')-Ia, *aac*(3)-IId, *aac*(6')-Ib-D181Y, *arr-3*, *dfrA27*, *aadA16*, *sul1* (2), *qnrB91*, *mph*(A), *tet*(A), *floR*, and *qnrS1* (Figure 1D). Notably, the plncFII_CTXM3_221-24 plasmid backbone was the most prevalent type carrying the *bla*_{CTX-M-3} gene in this study, with homologous sequences mapped in eight isolates. Comparative BRIG analysis using plncFII_CTXM3_218-74, plncFII_CTXM3_220-59, and plncFII_CTXM3_221-24 as references revealed conserved plasmid architectures among *bla*_{CTX-M-3}-positive isolates: (I) The plasmid from isolate 220-98 shared backbone structure and resistance gene composition with pIncFII_CTXM3_218-74; (II) Isolates 217-89 and 218-12 carried plasmids structurally aligned with pIncFII_CTXM3_220-59; (III) A

TABLE 2 Information about plasmid carrying *bla*_{CTX-M-3} or *bla*_{OXA-10} or *bla*_{CTX-M-14}^a.

Isolate	ST	Inc ^b	Plasmid length (bp)	Resistance genes carried by this plasmid	Resistance genes carried by isolate chromosomes	Other plasmids and the resistance genes
217-63	25	IncFII IncFIB	261642	<i>bla</i> _{OXA-10} , <i>bla</i> _{LAP-2} , <i>sul2</i> , <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>tet</i> (A), <i>floR</i> , <i>cmlA5</i> , <i>aadA1</i> , <i>dfrA14</i> , <i>qnrS1</i>	<i>bla</i> _{SHV-110} , <i>oqx</i> B17, <i>oqx</i> A7, <i>fosA6</i>	IncI1: <i>sul2</i> , <i>bla</i> _{CTX-M-14}
218-74	65	IncFII	119574	<i>bla</i> _{CTX-M-3} , <i>sul2</i> , <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>tet</i> (A), <i>floR</i> , <i>qnrS1</i>	<i>bla</i> _{SHV-11} , <i>oqx</i> B6, <i>oqx</i> A, <i>fosA6</i>	/
219-96	133	/	/	/	<i>bla</i> _{CTX-M-14} (2), <i>bla</i> _{SHV-75} , <i>qnrS1</i> , <i>oqx</i> B4, <i>oqx</i> A10, <i>fosA6</i>	IncFII: <i>bla</i> _{CTX-M-14} , <i>qnrS1</i>
220-59	792	IncFII	71104	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-1} , <i>qnrS1</i>	<i>bla</i> _{SHV-187} , <i>fosA</i> , <i>oqx</i> B19, <i>oqx</i> A9	/
221-24	967	IncFII	88594	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-1} , <i>sul2</i> , <i>aph</i> (3'')-Ib, <i>aph</i> (6)-Id, <i>aph</i> (3')-Ia, <i>aac</i> (3)-IId, <i>aac</i> (6')-Ib-D181Y, <i>arr-3</i> , <i>dfrA27</i> , <i>aadA16</i> , <i>sul1</i> (2), <i>qnrB91</i> , <i>mph</i> (A), <i>tet</i> (A), <i>floR</i> , <i>qnrS1</i>	<i>bla</i> _{SHV-27} , <i>oqx</i> B21, <i>oqx</i> A6, <i>fosA</i> , <i>sul2</i>	/

a) “/” means not applicable or blank.

b) Plasmid incompatibility group carrying *bla*_{CTX-M-3} or *bla*_{OXA-10} genes. The plasmids in isolates 217-63, 218-74, 220-59, and 221-24 were designated as plncFII+FIB_OXA10-217-63, plncFII_CTXM3_218-74, plncFII_CTXM3_220-59, and plncFII_CTXM3_221-24, respectively.

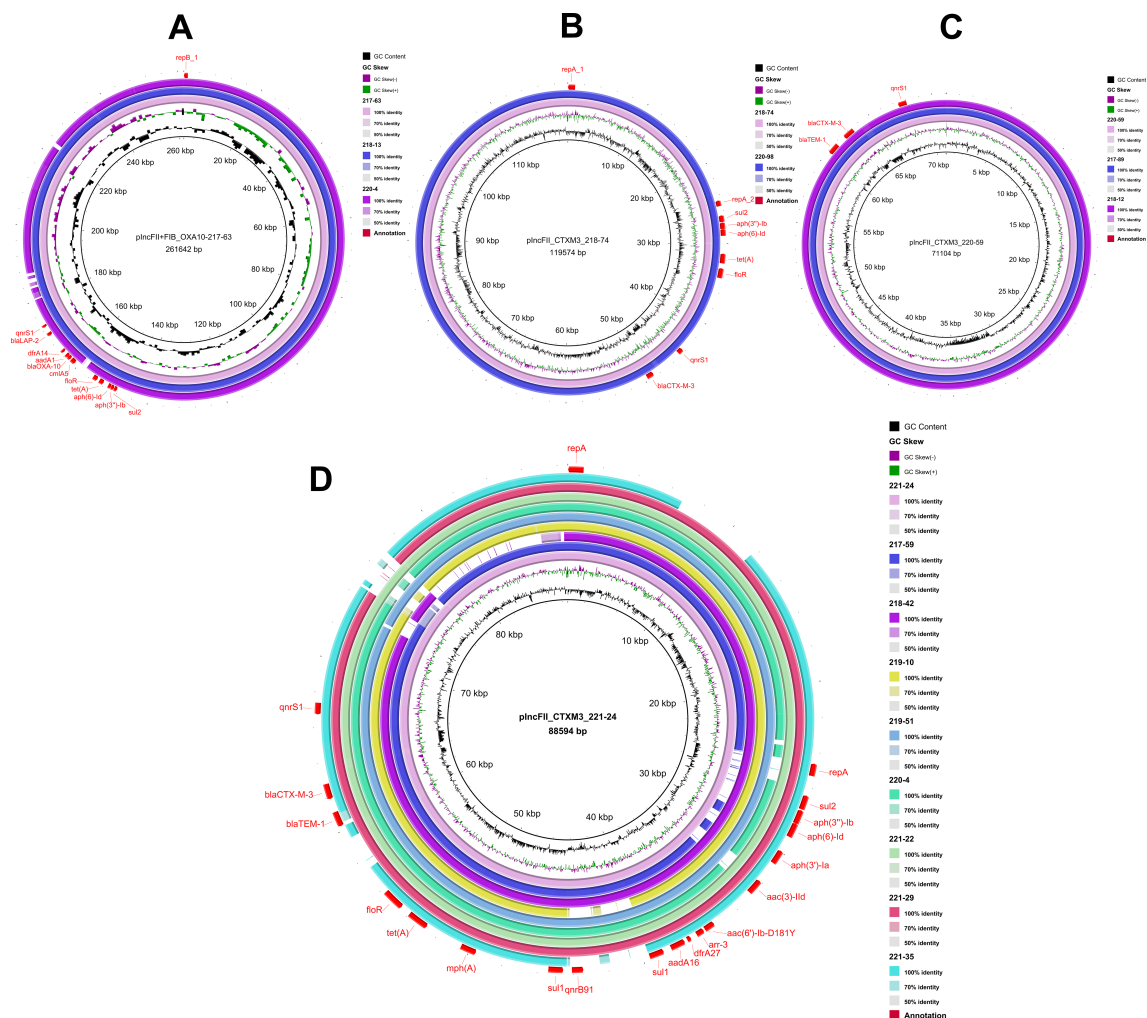


FIGURE 1

Plasmid backbone comparison. Using the corresponding plasmid sequence as a reference, the inner circle represents the GC content, while the second circle illustrates the GC skewness. The third circle depicts the sequence on the second-generation sequencing map of the isolate that carries the corresponding plasmid. Additional circles correspond to different isolates, which are identified by their logos located in the upper right corner of the figure. Annotations in the outermost circle highlight the locations of plasmid replicons and resistance genes, which are indicated in red font. (A), pIncFII-FIB_OXA10-217-63; (B), pIncFII_CTXM3_218-74; (C), pIncFII_CTXM3_220-59; (D), pIncFII_CTXM3_221-24.

cluster of eight isolates (217-59, 218-42, 219-10, 219-51, 220-4, 221-22, 221-29, 221-35) exhibited plasmids matching the genetic organization of the dominant pIncFII_CTXM3_221-24.

The genetic environment of *bla*_{CTX-M-3} and *bla*_{OXA-10} gene

The gene environments (Figure 2) of *bla*_{OXA-10} and *bla*_{CTX-M-3} were reconstructed using the extracted regions from four plasmids with known sequences, as illustrated in Figure 1. The genetic structure of *bla*_{OXA-10} is characterized by the arrangement “IS26-*cmlA5*-*bla*_{OXA-10}-*aadA1*-IS26” (Figure 2A). In contrast, the *bla*_{CTX-M-3} gene was associated with a diverse array of mobile genetic elements, including IS26, IS1X2, ISEcp1, ISKpn19, Tn2, and Tn3. Additionally, the *bla*_{TEM-1} gene is frequently found in close

proximity to *bla*_{CTX-M-3} (Figure 2B). Notably, the *bla*_{TEM-1} gene on plasmid pIncFII_CTXM3_218-74 is incomplete.

Phylogenetic analysis of *bla*_{CTX-M-3}-positive *K. pneumoniae* isolates

The complete sequences of 68 *bla*_{CTX-M-3}-positive *K. pneumoniae* genomes were downloaded from the NCBI Pathogen Detection Database (accessed on August 20, 2024). These genomes were analyzed alongside the 14 *bla*_{CTX-M-3}-positive *K. pneumoniae* isolates investigated in this study to assess their evolutionary relationships (Figure supplement 1). The figure presented key features of the isolates, including the STs, the type of plasmid Inc replicon, and associated resistance genes. The analysis revealed that *bla*_{CTX-M-3}-positive *K. pneumoniae* exhibited high genetic diversity,

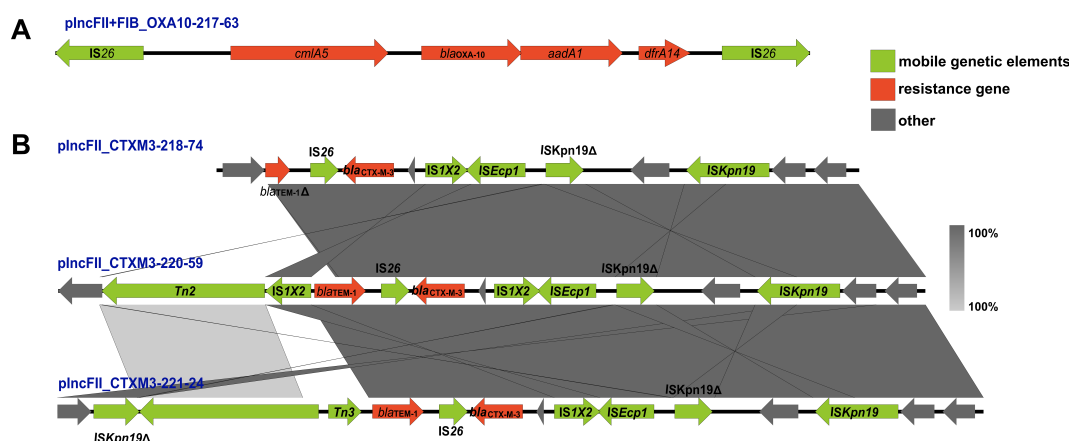


FIGURE 2

The genetic environment of *bla*_{CTX-M-3} and *bla*_{OXA-10} gene. Arrows of various colors represent distinct open reading frames (ORFs), with red indicating drug resistance genes, green denoting mobile genetic elements, and grey signifying other genes. The direction of the arrow illustrates the direction of transcription. Regions of sequence identity are highlighted by light grey shading. The symbol Δ indicates that the gene is incomplete. (A) depicts the genetic environment surrounding *bla*_{OXA-10} on plasmid pIncFII+FIB_OXA10-217-63, while (B) illustrates the genetic environment surrounding *bla*_{CTX-M-3} on plasmids pIncFII_CTXM3_218-74, pIncFII_CTXM3_220-59, and pIncFII_CTXM3_221-24.

encompassing over 40 distinct STs without a single dominant ST. Interestingly, the majority of *bla*_{CTX-M-3}-positive isolates were found to harbor IncFII plasmid replicons and frequently carried TEM-type and SHV-type resistance genes.

Discussion

The prevalence of *K. pneumoniae* isolates exhibiting peculiar resistance phenotype in this study highlights the complexity of antimicrobial resistance in this pathogen. FEP, a fourth-generation cephalosporin representative, is widely used for treating infections caused by GNB (Endimiani et al., 2008; Shoji et al., 2016). Due to its enhanced stability against hydrolysis by extended-spectrum β-lactamases (ESBLs), it typically exhibits superior *in vitro* activity compared to third-generation cephalosporins (Fung-Tomc et al., 1989; Okamoto et al., 1994). Paradoxically, our clinical observations revealed an anomalous resistance phenotype in some *K. pneumoniae* isolates, showing reduced susceptibility to FEP compared to CAZ—a phenomenon we designated as the “peculiar resistance phenotype”. The 20 *K. pneumoniae* strains analyzed displayed significant genetic diversity, with 10 distinct STs identified, including ST25 (4/20), ST967 (3/20), ST65 (2/20), ST133 (2/20), ST48 (2/20), ST353 (1/20), ST628 (1/20), ST753 (1/20), ST792 (1/20), and ST254 (1/20). Notably, several of these ST types, such as ST25, ST967, and ST65, have been previously reported in clinical isolates (Yu et al., 2018). Notably, no single ST was dominant among the isolates, suggesting that the peculiar resistance phenotype is not restricted to a specific *K. pneumoniae* clonal lineage. Phylogenetic analyzed comparing the strains in this study with those retrieved from NCBI further supported this observation.

In this study, all isolates demonstrated resistance to FEP while remaining susceptible or exhibiting intermediate susceptibility to

CAZ. Among these isolates, the presence of the *bla*_{CTX-M-3} gene in 14 strains is a key contributor to high-level resistance to FEP. CTX-M-3 is a member of the CTX-M type of ESBLs, first reported in 1998 in strains of *Citrobacter freundii* and *Escherichia coli* from a Polish hospital (Gniadkowski et al., 1998). Currently, it has been identified in various *Enterobacteriaceae*, including *K. pneumoniae*, *Enterobacter cloacae*, and *Morganella morganii* (Moriguchi et al., 2007; Furlan et al., 2020; Luo et al., 2020; Souna et al., 2022). However, it appears that prior to this study, the potential of the *bla*_{CTX-M-3} gene to facilitate the emergence of peculiar resistance phenotype had not been adequately recognized. The conjugation experiments and associated data presented herein demonstrate that the IncFII plasmid carrying *bla*_{CTX-M-3} can mediate the development of higher-level peculiar resistance phenotype. In contrast, the role of *bla*_{CTX-M-14} appears to be more restricted. The mechanisms driving resistance in *P. aeruginosa* differed markedly from those in *K. pneumoniae*. In *P. aeruginosa*, the mexXY-OprM efflux system plays a central role, supplemented by various β-lactamases, including *bla*_{OXA-1}, *bla*_{OXA-10}, *bla*_{OXA-31}, *bla*_{OXA-35}, and *bla*_{PSE-1} (Aubert et al., 2001; Hocquet et al., 2006; Campo Esquisabel et al., 2011). Notably, *bla*_{OXA-10} was identified in only a limited number of strains in this study.

Mobile genetic elements, including plasmids, transposons, and integrons, are crucial drivers of the horizontal transfer of antibiotic resistance genes among bacterial strains (Su et al., 2018; Tran et al., 2021). The *bla*_{CTX-M-3} gene is predominantly located on an IncFII plasmid in this study. This plasmid is recognized for its high mobility and capacity to transfer resistance genes across various bacterial species (De Koster et al., 2022). In addition to *bla*_{CTX-M-3}, the IncFII plasmid harbors multiple other resistance determinants, thereby contributing to multidrug resistance in the host bacterium. The presence of such a plasmid significantly enhances the adaptability and survival of resistant strains, rendering them difficult to treat

with commonly used antibiotics. This study identified three distinct IncFII plasmid backbones carrying *bla*_{CTX-M-3}, represented by pIncFII_CTXM3_218-74, pIncFII_CTXM3_220-59, and pIncFII_CTXM3_221-24. Among these, pIncFII_CTXM3_221-24 was the most prevalent. In addition to *bla*_{CTX-M-3}, pIncFII_CTXM3_221-24 carries a variety of other resistance genes, including *bla*_{TEM-1}, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *aph(3')-Ia*, *aac(3)-IId*, *aac(6')-Ib-D181Y*, *arr-3*, *dfrA27*, *aadA16*, *sul1* (2), *qnrB91*, *mph(A)*, *tet(A)*, *floR*, and *qnrS1*. These genes confer resistance to a broad range of antibiotics, including sulfonamides, aminoglycosides, tetracyclines, and quinolones, further complicating treatment strategies for infections caused by these strains. Given the potential of plasmids for widespread dissemination and their role in the emergence of resistant pathogens, it is essential to closely monitor the spread of IncFII plasmids in clinical settings to mitigate the impact of antibiotic resistance. Furthermore, the *bla*_{CTX-M-3} gene was frequently associated with mobile genetic elements such as IS26, *ISEcp1*, and Tn3, which are well-documented facilitators of gene mobilization and horizontal transfer (Bevan et al., 2017; Partridge et al., 2018). The proximity of these elements to *bla*_{CTX-M-3} supported the hypothesis that these genes could be rapidly disseminated within bacterial populations, significantly contributing to the spread of resistance. Moreover, the co-location of *bla*_{CTX-M-3} with other resistance genes, including *bla*_{TEM-1}, highlighted the complexity of resistance gene clusters in *K. pneumoniae*. This arrangement increased the potential for co-selection of resistance traits, particularly under high antibiotic pressure, where multiple resistance determinants might be selected simultaneously. In the case of *bla*_{OXA-10}, the integron gene cassette array “*aadA1-bla*_{OXA-10}-*cmlA5-arr2-dfrA14*” had been identified in various plasmids associated with several *Enterobacteriaceae* species, indicating its widespread dissemination (Arpin et al., 2012; Suzuki et al., 2019). Similar resistance genes were present around *bla*_{OXA-10} in this study. The presence of the composite transposon structure “IS26-*cmlA5-bla*_{OXA-10}-*aadA1*-IS26” in this study suggested that this gene resided within a relatively stable genetic framework. This stability likely enhanced its persistence within the *Klebsiella pneumoniae* and facilitated its propagation (Harmer and Hall, 2016).

Our study provided valuable insights into the molecular mechanisms underlying the peculiar resistance phenotype observed in *K. pneumoniae* strains. Clinicians should be aware that certain *K. pneumoniae* strains may harbor β -lactamase genes capable of conferring differential resistance to specific cephalosporins, posing potential challenges in treatment decision-making, especially in the context of empiric therapy. Thankfully, while resistance to β -lactam antibiotics, such as FEP, the strains retained susceptibility to carbapenems (IPM and ETP) and aminoglycosides (AMK). This susceptibility pattern of resistance underscored the critical role of carbapenems as the last-resort therapy for MDR Gram-negative infections (Liu et al., 2022; Wu et al., 2022). In summary, this study enhanced our understanding of

the complex resistance mechanisms in *K. pneumoniae*, with a specific emphasis on the pivotal roles of *bla*_{CTX-M-3} and *bla*_{OXA-10}. The emergence of peculiar resistance phenotype underscores the urgent need for ongoing monitoring and the development of novel therapeutic strategies to address the growing threat posed by multidrug-resistant *K. pneumoniae*.

Conclusion

This study identified a peculiar resistance phenotype in *K. pneumoniae*, characterized by reduced susceptibility to cefepime compared to ceftazidime, accompanied by a diverse range of STs. The absence of a dominant ST indicated that this resistance phenotype was widely distributed across diverse clonal populations. Further analysis revealed that the primary driver of this phenotype is the presence of the IncFII plasmid carrying the *bla*_{CTX-M-3} gene. This plasmid not only harbored multiple additional resistant genes but also demonstrated high conjugation and transferability, facilitating the dissemination of resistant genes. Moreover, some strains possessed an IncFII/IncFIB fusion plasmid that contains the *bla*_{OXA-10} gene, which further enhances resistance. This resistance mechanism in *K. pneumoniae* shows significant differences compared to *P. aeruginosa*. These findings underscore the critical role of specific plasmids in the development of the resistance phenotype in *K. pneumoniae*, which could complicate the treatment of infections. Clinicians may need to consider alternative antibiotics or combination therapies, and surveillance for such resistant strains should be prioritized to guide effective treatment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

SZ: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. FZ: Conceptualization, Supervision, Writing – review & editing. PL: Formal analysis, Methodology, Writing – original draft. LY: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. JS: Formal analysis, Writing – review & editing. CL: Methodology, Writing – review & editing.

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Conflict of interest

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

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1545157/full#supplementary-material>

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In vivo RNA-seq and infection model reveal the different infection and immune characteristics of *B. pertussis* strains in China

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Introduction: Various strains emerged in *B. pertussis* re-emergence, the pathogenic characteristics and mechanisms remain elusive. We aimed to explore the relationship between the *in vivo* transcriptome and colonization advantage of various pertussis clinical strains during the *B. pertussis* re-emergence.

Methods: Four pertussis strains were isolated from clinically suspected cases by active surveillance. The phylogenetic relationships of clinical strains and global isolates were compared by a genome-wide SNP-based phylogenetic tree and allele genotyping. LC-MS/MS analysis and binding affinity detection allowed the identification of expression and antigenicity of pertactin. The characteristics of infection and immunity of clinical strains were compared in a BALB/c mouse aerosol challenge model. *In vivo* RNA-seq analysis was performed in NSG mouse model to describe the transcriptome during infection, and verified by detecting biofilm formation and paraquat tolerance.

Results: The partial pertactin-deficient strain BP-L2 was first reported. It showed significantly enhanced tracheal colonization compared to both CS and BP-L1 strains in naive mice ($P < 0.0001$ vs. CS) and exhibited superior fitness over BP-L1 in immunized mice ($P < 0.001$). BP-L1 showed superior lung colonization ($P < 0.0001$) and tissue-resident memory T cell induction versus BP-L2 and CS ($P < 0.001$). Colonization dominance of BP-L1 in lungs and BP-L2 in trachea aligned with the pathological injury ($P < 0.05$) and the inflammatory cytokine enhancement (IL-6 in lungs of BP-L1 group, $P < 0.01$). *In vivo* RNA-seq results revealed that BP-L2 significantly upregulated *relA* ($\log_2FC = 2.1$, $FDR P\text{-value} =$

0.019) and *sodA* ($\log_2FC = 2.4$, $FDR\ P\text{-value} = 8.61E-06$) compared to BP-L1, functionally linked to enhanced stringent response and oxidative stress defense. BP-L1 exhibited significant *in vivo* *bipA* upregulation over BP-L2 ($\log_2FC = 1.8$, $FDR\ P\text{-value} = 0.027$) without concurrent biofilm enhancement ($P = 0.51$ vs. BP-L2). Furthermore, the BP-L2 and BP-L3 strains of the same *ptxP1-ptxA1-fhaB3* lineage showed significantly higher paraquat tolerance than other strains ($P < 0.001$), showing extremely high SODs activity.

Conclusion: The emerging pertussis strains exhibit different colonization advantages in the trachea or lungs, which will influence the transmission patterns of the clinical strains. The tracheal colonization advantage of the partial pertactin-deficient strain may be associated with the overexpression of the *relA* and *sodA* *in vivo* infection.

KEYWORDS

Bordetella pertussis, *in vivo* RNA, clinical strains, tracheal colonization, pertactin deficiency

Introduction

Bordetella pertussis (*B. pertussis*) is the major causative agent of pertussis, a highly contagious, acute respiratory disease in humans. Since the 1980s, there has been a significant increase in the global incidence of pertussis, known as re-emergence of pertussis, which has become a serious public health problem (Burns et al., 2014). China introduced the combined diphtheria-tetanus-whole-cell pertussis (DTwP) vaccine in 1978. In 2007, the combined diphtheria-tetanus-acellular pertussis (DTaP) vaccine was incorporated into the Expanded Program on Immunization (EPI), gradually replacing DTwP until the transition was completed in 2010 (Meng et al., 2018b). The current immunization schedule of China administers the primary DTP series at 2, 4, and 6 months of age, followed by booster doses at 18 months and 6 years of age and the coverage for the three primary doses exceeds 90% (Meng et al., 2018a). However, there has also been an increasing incidence of pertussis in China since 2013, peaking in 2024 (National Disease Control and Prevention Administration, 2025). The re-emergence of pertussis may be related to various factors, including increased awareness, improved diagnostic methods (Belcher and Preston, 2015), suboptimal vaccination (Diavatopoulos and Edwards, 2017), waning vaccine-induced immunity (Klein et al., 2012) and pathogen adaptation (Ma et al., 2021). However, waning immunity of acellular pertussis vaccine (aP) and pathogen adaptation are considered to be important factors affecting the resurgence of *B. pertussis*.

In recent epidemiological studies of pertussis conducted worldwide, a large number of new *B. pertussis* epidemic strains were discovered. The latest *B. pertussis* epidemic strains exhibit multiple antigen alleles with changes, antigen deficiency and other characteristics (Octavia et al., 2012; Ma et al., 2021) under vaccine selection pressure. The re-emergence of pertussis was consistent

with the emergence of strains harboring a specific allele of the pertussis toxin operon promoter, *ptxP3*, which has been reported to be associated with increased pertussis toxin expression (Mooi et al., 2009). Notably, *ptxP3* strains have spread globally, as they have emerged in several countries in Europe (Mooi et al., 2009), North America (Schmidtke et al., 2012) and Australia (Octavia et al., 2012). Furthermore, studies have reported a dominance of the *bscI3* allele in the *ptxP3* lineage, which is associated with decreased type III secretion system and may allow *B. pertussis* to reduce immune recognition (Xu et al., 2022). The *fhaB* allele associated with higher mutation rates was also found in the *ptxP1* lineage in China, which may be associated with vaccine escape (Xu et al., 2019). However, the *ptxP3* strains were still the dominant type in China and most of them were macrolide resistant strains carrying the 23sRNA A2047G mutation (Fu et al., 2023).

In addition, vaccine-associated antigen protein-deficient strains, such as those lacking pertussis toxin (PT), filamentous hemagglutinin (FHA) and pertactin (PRN), have also been identified among the currently dominant isolates. However, pertussis strains lacking additional vaccine antigens have been identified (Bouchez et al., 2015; Williams et al., 2016), though their prevalence remains much lower than that of PRN-deficient isolates (Ma et al., 2021). This fact highlights critical gaps in understanding both the biological role and immunological consequences of PRN during *B. pertussis* infection.

The *ptxP3* allele and PRN-deficient strains are predominant in countries with high vaccination rates (Zhao et al., 2021) and may compromise the protective efficacy of aP vaccines. Although the allele profiles of current epidemic strains differ from historical variants, very few studies have evaluated the infection characteristics and immune protection induced by the current clinical strains (Mooi et al., 2009; Safarchi et al., 2015). The relationship between strain-

specific colonization/immune adaptations and transmission efficiency remains unclear. Furthermore, with the development of methods to isolate *B. pertussis* in the infected state *in vivo* (Wong et al., 2019), the relative contributions of allelic variation versus infection-phase gene regulation to phenotypic differences require further investigation.

Previous studies have demonstrated that specific gene functions during *B. pertussis* infection facilitate bacterial colonization within distinct locations of the host respiratory tract (Belcher et al., 2021). For example, the adenylate cyclase toxin-hemolysin (ACT) facilitates early stages of airway colonization by disrupting the phagocytic clearance (Espinosa-Vinals et al., 2025); a study using MyD88-deficient C57BL/6J mice—a model replicating human catarrhal-phase infection—demonstrated that *FhaB* and *Fim* are essential for pertussis nasal colonization (Holubova et al., 2022). In addition, a study found the sigma factor *RpoN* of *Bordetella bronchiseptica* is involved in bacterial motility and initial biofilm formation, and is essential for tracheal colonization (Ma et al., 2024). The above studies emphasize that the analysis of the expression of key genes during bacterial infection will help discover the mechanisms that cause different infection colonization characteristics of pertussis strains.

In this study, whole-genome sequencing was conducted for all four isolated strains (BP-L1 to BP-L4), which were isolated from 158 clinically suspected cases in the Xishuangbanna region of Yunnan Province, China. Phylogenetic analysis revealed that the four isolates were divided into two major clades, with BP-L2 identified as a partial PRN-deficient strain. Combined analysis of post-infection antibodies, antibody binding affinity and LC-MS/MS confirmed the expression of a partially defective PRN in BP-L2. Subsequently, the pertussis aerosol challenge in mice revealed that BP-L1 and BP-L2 had significantly increased colonization in the lungs and trachea ($P < 0.0001$ vs. other strains), respectively, and caused pathological injury and elevated inflammatory cytokines at the corresponding locations. BP-L1 infection induced the highest proportion of lung tissue-resident memory T (Trm) cells ($P < 0.001$), whereas BP-L2 exhibited superior fitness in immunized hosts ($P < 0.001$ vs. BP-L1). *In vivo* RNA-seq revealed BP-L2-specific upregulation of stringent response ($\log_2FC = 2.1$, FDR P -value = 0.019) and antioxidant stress genes ($\log_2FC = 2.4$, FDR P -value = $8.61E-06$) over the BP-L1. This oxidative stress tolerance was functionally validated through paraquat challenge, with BP-L2 and BP-L3 within the same *ptxP1-ptxA1-fhaB3* lineage demonstrating higher survival rates to other strains ($P < 0.001$). Overall, for the first time, we explored the different infection characteristics and possible underlying mechanisms of various pertussis clinical strains by combining aerosol infection model and *in vivo* RNA-seq results.

Materials and methods

Ethics approval statement

This study received ethical approval from the People's Hospital of Xishuangbanna Dai Autonomous Prefecture Ethics Committee

(Approval No. 1706001x) with written informed consent from all participants, whose privacy rights were strictly protected. Animal experiments were conducted under specific pathogen-free conditions at the Institute of Medical Biology, Chinese Academy of Medical Sciences (IMBCAMS), in compliance with protocols approved by the Institutional Animal Care and Use Committee (Approval Nos. DWSP202011004 and DWSP201911014).

B. pertussis strains and vaccine

Between October 2018 and October 2021, nasopharyngeal swabs were collected from 158 suspected pertussis cases in Xishuangbanna Dai Autonomous Prefecture. All samples were spread onto Reagan-Lowe agar plates (Oxoid, Thermo Fisher Scientific, Waltham, United States) supplemented with 15% defibrinated sheep blood (Beijing Minhai Biotechnology Co., Ltd, Beijing, China) and Bordetella selective supplement (Oxoid), and cultured at 37°C for 3–7 days. Colonies exhibiting characteristic morphology (diameter 0.5 – 1.0 mm, mercury-drop appearance) were confirmed by the slide agglutination test with *B. pertussis* and *Bordetella parapertussis* antisera (Remel Europe Ltd., UK) and subsequent PCR confirmation as stated in the previous study (Wu et al., 2019). Four *B. pertussis* strains were successfully isolated from 158 samples (isolation rate: 2.53%) and designated BP-L1 through BP-L4. Regarding the vaccine, the antigens in the aP vaccine we used were detoxified using glutaraldehyde and subsequently adsorbed onto aluminum hydroxide individually. Each dose of the aP vaccine contains 25 µg of PT, 25 µg of FHA and 8 µg of PRN. The aP vaccine was produced by the Institute of Medical Biology, Chinese Academy of Medical Sciences (IMBCAMS) under good manufacturing practice conditions (Sun et al., 2014) and then adjusted to 1/40 human dose for subsequent use.

Whole-genome sequencing

All four *B. pertussis* clinical isolates were subsequently subcultured on Bordet-Gengou agar plates supplemented with 15% defibrinated sheep blood and without cephalexin, incubated at 37°C for 72 hours. The resulting colonies were hemolytic and less than 1.0 mm in diameter, resembling mercury droplets and glistening. Then, DNA was isolated using a QIAGEN Genomic-tip 100/G kit (10243, Qiagen, Hilden, Germany) following the manufacturer's instructions. The whole-genome sequencing process was performed according to the standard protocol provided by Oxford Nanopore Technologies. Bacterial genomic DNA was subjected to rigorous quality assessment using a Nanodrop One spectrophotometer (Thermo Fisher Scientific), a Qubit 4.0 Fluorometer (Thermo Fisher Scientific) and 0.35% agarose gel electrophoresis. Large DNA fragments (>20 kb) were size-selected using the BluePippin automated nucleic acid recovery system (Sage Science) with 0.75% agarose cassettes. Sequencing libraries were prepared with the SQK-LSK109 ligation sequencing

kit (Oxford Nanopore Technologies). Final libraries were quantified via Qubit 4.0 Fluorometer and loaded onto FLO-MIN106D flow cells for sequencing on a MinION platform (Oxford Nanopore Technologies) using MinKNOW v22.05.0 software for real-time data acquisition. Raw fast5 data were base-called to fastq format using Guppy v3.2.6 integrated within the MinKNOW software package. Adapter sequences, low-quality reads (Q-score <7), and short fragments (<2000 bp) were filtered out using Porechop v0.2.4 and Filtlong v0.2.1. Filtered subreads were assembled into draft genomes with Canu v1.5. Final assemblies were annotated with Prokka v1.14.6, and all genomes achieved a minimum coverage depth of 50×, validated through alignment statistics and circular completeness checks. The SNPs, insertions and deletions were detected by mapping the contigs to the previously sequenced and annotated *B. pertussis* Tohama I genome using BLAST and were filtered as described in previous studies (Holt et al., 2008). The complete genomes of 84 pertussis isolates, used to analyze phylogenetic relationships were obtained from databases [PRJNA770762, PRJNA279196, PRJNA1178746, PRJNA695314, PRJNA530108] or recent studies (Bart et al., 2014; Boinett et al., 2015; Alai et al., 2022; Koide et al., 2022; Fu et al., 2023). The detailed strain information is provided in Supplementary Table 1. The assembled whole genome was aligned using snippy v3.1, with Tohama I as the reference genome, and the resulting phylogenetic tree was constructed using tvBOT (Xie et al., 2023). Allelic typing corresponds to the sequence of the Tohama I strain obtained from GenBank. The analysis was conducted using SeqKit v2.9.0, including only the exact sequences. Additionally, IS Finder was utilized to detect Insertion Sequence (IS) elements within the genomes (Park et al., 2012).

PRN purified by anion exchange chromatography

Referring to the previously reported methods (Li et al., 2016), after three subcultures at 36°C and 140 rpm for 48 hours in Stainer and Scholte medium (SS medium), pertactin from *B. pertussis* strains CS, BP-L1 and BP-L2 was initially extracted through heat treatment. Cells were re-suspended in five times the volume of PBS. The sample was centrifuged to remove the supernatant and other contaminating proteins. The suspension was incubated at 60°C for 3 hours and then centrifuged at 4°C, 8000 rpm for 15 minutes to remove bacterial cells. The supernatant was further concentrated to approximately 1/10 of its initial volume using ultrafiltration. The tangential-flow ultrafiltration module Vivaflow 200 (Sartorius, Germany), which consists of polyethersulfone with a molecular weight cutoff of 3 kDa, was used for ultrafiltration. The concentrated extract solution was dialyzed against 20 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 7.8 (buffer A) at 4°C. The sample was subsequently centrifuged to eliminate any insoluble impurities prior to chromatographic purification. Anion exchange chromatography was utilized for the capture step, employing Capto adhere (Cytiva, Cat. No. 17544401, USA). Approximately 25 mL of Capto adhere was packed into a column. The column was

equilibrated with 10 column volumes (CVs) of buffer A, followed by an additional wash with 2 CVs of the same buffer post-sample loading to remove unbound proteins. A step-gradient concentration of NaCl in buffer A was used, with 0.1 M for eluting the target protein and 1 M for regenerating the column. The flow rate was maintained at 2 mL/min throughout the process. Purification was conducted using the ÄKTA explorer 100 (GE Healthcare, USA), with detection of ultraviolet absorbance at 280 nm. For BP-L1 and CS strains, peak components emerging after approximately 2 CVs were collected, whereas all peak components from BP-L2 strains were recovered and combined as samples for testing.

LC-MS/MS analysis

Load the prepared PRN samples of various *B. pertussis* strains and protein markers into the wells of a 12% separating gel. Initiate electrophoresis at 80 V for 15 minutes, then increase the voltage to 120 V for an additional hour. Stain the gel with Coomassie Blue dye, excise three gel slices from each gel, and cut them into 1 mm³ cubes. Briefly centrifuge to pellet the slices and wash with 500 µL of 50 mM ammonium bicarbonate/acetonitrile (1:1, v/v) until destained. Remove the supernatant, dehydrate with 500 µL acetonitrile for 10 minutes. Rehydrate the slices in 10 mM DTT/50 mM ammonium bicarbonate at 56°C for 1 hour and repeat the acetonitrile dehydration. Treat the gel slices with 50 mM IAA/50 mM ammonium bicarbonate at room temperature for 1 hour in the dark, and then perform a final acetonitrile dehydration. Add enzyme digestion solution to cover the slices, incubate on ice for 45 minutes, and then add 5–20 µL of digestion solution to maintain hydration during the overnight incubation at 37°C. Collect the supernatant into a fresh tube, extract peptides with 100 µL of 50 mM ammonium bicarbonate/acetonitrile (1:2, v/v, 37°C, 1 hour), combine the extracts, and lyophilize to near dryness. Resuspend the peptides in 10 µL of 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS was performed on a Q ExactiveTM Hybrid Quadrupole-OrbitrapTM mass spectrometer (Thermo Scientific) coupled with an Easy-nLC 1200 system. For each sample, 5 µL was loaded onto a C18 PepMap100 trap column (300 µm × 5 mm) and separated on a Thermo Acclaim PepMap RPLC analytical column (150 µm × 15 cm) using a 66 min gradient: 4–8% B (2 min), 8–28% B (43 min), 28–40% B (10 min), 40–95% B (1 min), 95% B (10 min) (mobile phase A = 0.1% formic acid in water; B = 20% 0.1% formic acid/80% acetonitrile) at a flow rate of 0.6 µL/min. Data-dependent acquisition mode was employed with full MS scans (300–1800 m/z) at 70,000 resolutions (AGC target 3×10⁶ ions, max IT 100 ms) followed by top 15/20 MS/MS scans (HCD, 28% NCE) at 17,500 resolution (AGC target 1×10⁵ ions, max IT 50 ms), using a 3 s cycle time.

The raw MS files were analyzed and searched against target protein database based on the species of the samples using Byonic (v4.2.4 from Protein Metrics, Cupertino, CA). The mass tolerance was set to 20 ppm and 0.02 Da for the precursor and the fragment ion respectively, with up to two missed cleavages allowed. Carbamidomethyl was used as a fixed modification and oxidation

(M) (variable), Acetyl (Protein N-term) (variable) were used as a variable modification. Enzyme specificity was defined as chymotrypsin, trypsin, and Glu-C (Junker et al., 2006; Liang et al., 2024).

The binding affinities test by microscale thermophoresis

The isolated strains BP-L1, BP-L2, and CS *B. pertussis* were harvested in liquid SS medium. The PRN antigen was purified by column chromatography (Cytiva, USA). Rabbit Anti-*B. pertussis* pertactin antiserum (cat. #PRN11-S, Alpha Diagnostic International, San Antonio, TX) was used as the ligand. The binding affinities between PRN and ligands were determined according to the manufacturer's protocol for the MO RED-NHS Kit (Sedivy, 2021) (NanoTemper Technologies GmbH, Munich, Germany).

B. pertussis aerosol challenge and vaccines programs

The *B. pertussis* strains utilized for the aerosol challenge were BP-L1 and BP-L2, which were isolated from clinical cases within this study. Additionally, the *B. pertussis* strain CS was included as a reference strain (Zhang et al., 2011). *B. pertussis* infection was induced through aerosol challenge with a concentration of 10^{11} CFU/mL, administered via a nebulizer for 30 minutes (Jiang et al., 2021). A total of 132 four-week-old BALB/c mice, used in this study, were procured from Vital River Laboratory Animal Technology Co., Ltd. As previously described, the progression of the infection was monitored by counting colony-forming units (CFU) in lung and tracheal homogenates and nasal lavage at various intervals post-infection (p.i.) (McGuirk et al., 1998). Specifically, plates with CFU counts ranging from 30 to 300 were selected for counting, and the number of viable colonies was calculated based on the dilution factor. Four weeks post *B. pertussis* challenge, serum PRN-antibody levels were quantified using ELISAs. Furthermore, a total of 52 BALB/c mice, with an equal number of females and males, were vaccinated with a 1/40 human dose of the aforementioned aP vaccine (Zuo et al., 2021). Two doses were administered at 3-week intervals. All vaccinations were conducted under isoflurane anesthesia. Six weeks post-immunization, 48 mice were randomly divided into two groups for respiratory challenge with the BP-L1 strain or BP-L2 strain.

Tissue-resident memory T cell assay

The levels of tissue-resident memory T cells (Trm) in the lungs of mice from different groups were examined 9 weeks following respiratory challenge and 9 weeks post-aP vaccine immunization. The method has been detailed in previous research (Wilk et al., 2017). In brief, we administered an anti-mouse PE-CD45 Ab

(eBioscience, San Diego, CA) intravenously to mice 10 minutes before they were euthanized, and the lung lymphocytes were isolated for subsequent flow cytometry, as previously described (Wilk et al., 2017). Flow cytometric analysis was performed on a Beckman Coulter CytoFLEX, and the results were analyzed using CytExpert v2.4.0.28.

B. pertussis in vivo and in vitro RNA-seq

B. pertussis cells from the *in vivo* infection state were isolated and subjected to *in vivo* RNA-seq. The specific methods used are detailed in the previous study (Wong et al., 2019). Specifically, 12 NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice, consisting of an equal number of males and females, were purchased from Shanghai Model Organisms Center, Inc., to increase the lung bacterial burden (Wong et al., 2019). The aerosol challenge of NSG mice was conducted using the same procedure as that for BALB/c mice. Specifically, *B. pertussis* infection was induced via aerosol challenge (10^{11} CFU/mL) by administering a nebulizer for 30 minutes. Each of the BP-L1, BP-L2, and CS bacterial strains infected 4 NSG mice, with an equal number of males and females in each group. The lungs and trachea were extracted on the seventh day post-infection. *B. pertussis* cells were concentrated using a 70- μ m-pore-size cell strainer (VWR), and the filtrate was subsequently passed through a 5- μ m-pore-size filter (Minisart type 17594 cellulose acetate; Sartorius). The bacteria were then pelleted by final centrifugation at 4°C at $16,100 \times g$ (Wong et al., 2019). For *in vitro* RNA sequencing analysis, *B. pertussis* strains were grown in SS liquid medium for 24 hours at 36°C and harvested for transcriptome sequencing.

For the RNA sequencing, rRNA was removed from total RNA using a Ribo-Zero rRNA Removal Kit Bacteria (Illumina, San Diego, CA). RNA-seq was performed on the Illumina NovaSeq 6000 platform. The reads were aligned to the *B. pertussis* Tohama I genome using Bowtie2 v2.2.8. DEGs were calculated using the edgeR package, with a difference fold threshold of 2 and an FDR-adjusted *P* (q value) of less than 0.05. The *B. pertussis* genome contains numerous repeated insertion sequences (IS), and to prevent errors from random mapping, all IS-related transposase reads were excluded from the analysis. Since 99% of the reads in the *in vivo* RNA sequencing data originated from the host, we increased the sequencing depth to 150 million 2×150-bp paired-end reads to ensure adequate coverage of *Bordetella pertussis*-derived sequences.

Biofilm formation assay

Following the methods of previous studies (Držmíšek et al., 2023), *B. pertussis* cells from CS and BP-L1 to BP-L4 were suspended in liquid SS media to achieve a final OD600 of 0.3. Triplicate samples of the diluted cultures (200 μ L) were inoculated in parallel into 96-well non-treated tissue culture plates. The plates were incubated at 37°C for 72 hours. Loosely adherent and planktonic bacteria were removed by washing the wells three times with PBS. Adherent cells were stained

with a 0.1% crystal violet solution for 45 minutes. The plates were then washed with PBS and dried, after which the adsorbed crystal violet was solubilized in 200 μ L of 95% ethanol for 15 minutes. To quantify biofilm formation, the absorbance at A₅₉₅ of the crystal violet staining was measured using a microplate reader (Thermo Fisher). The readings from the wells were adjusted against the substrate blank well, and each value was the average of the assays, with two replicates per assay. Each experiment was repeated three times.

Paraquat sensitivity assay

Adopting the methods from previous studies (Graeff-Wohlleben et al., 1997; Martins et al., 2018), the *B. pertussis* strains CS and BP-L1 to BP-L4 were cultured overnight in liquid SS medium. The bacterial cultures were then diluted in PBS to an optical density of OD₅₉₀ = 0.1, and the CFU for each strain were determined on Reagan-Lowe agar plates supplemented with 15% defibrinated sheep blood. The entire 5 g of paraquat powder (Sigma) was dissolved in 50 mL of PBS, followed by sterile filtration and the transfer of 1,285 μ L of the paraquat solution into 10 mL of bacterial suspension to achieve a final concentration of 50 mM. The samples were subsequently shaken at 37°C. After 30 minutes, the samples were diluted again, and the CFU was determined on the same Reagan-Lowe plates. The percentage of surviving bacteria for each strain was calculated, with each experiment being repeated three times.

Statistical analysis

All data are expressed as the mean $\pm$ SEM and were analyzed using GraphPad Prism v9.0 (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) was utilized to assess the statistical significance of differences among three or more groups. A value of $P < 0.05$ was considered to indicate statistical significance. For transcriptome analysis, Bonferroni-corrected q values of less than 0.05 were deemed statistically significant.

Results

Whole-genome sequence characteristics of clinical strains

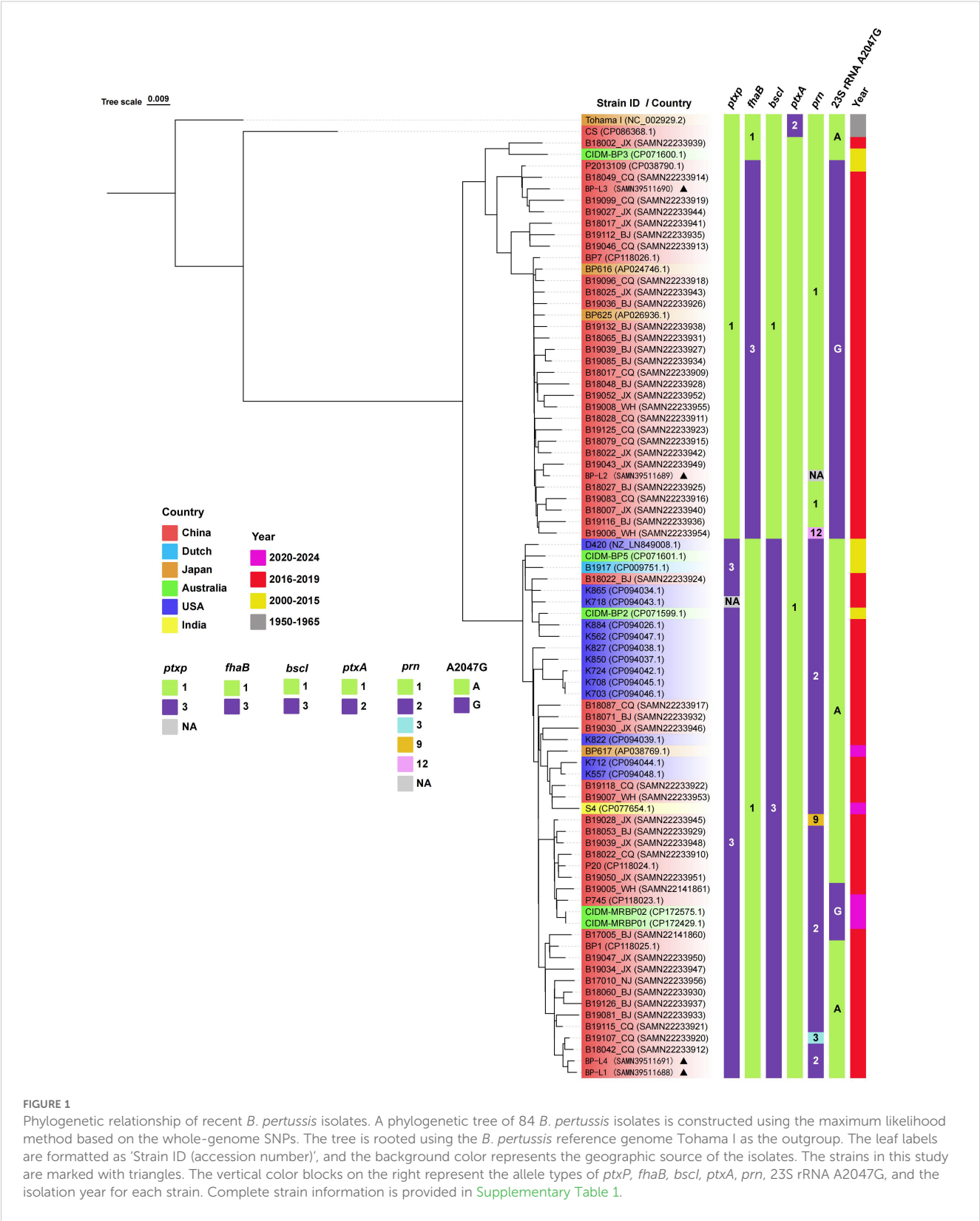
Whole-genome sequencing was conducted on all four clinical isolates in this study. Following rigorous quality assessments and data cleaning, between 60,262 and 111,052 sequence reads were obtained for BP-L1 to BP-L4, with mean quality scores ranging from 8.98 to 9.27 (refer to Supplementary Table 2). Allelic genotyping indicated that BP-L1 and BP-L4 possessed the *ptxP3/ptxA1/prn2/fhaB1/bscI3* genotype; BP-L3 had the *ptxP1/ptxA1/prn1/fhaB3/bscI1* genotype, and BP-L2 exhibited the *ptxP1/ptxA1/fhaB3/bscI1* genotype. The phylogenetic relationships among 82 recent *B. pertussis* isolates and 2 reference strains are depicted in

Figure 1. These strains were sourced from China, the USA, Australia, Japan, India, and the Netherlands, and were isolated between the years 2000 and 2024. The phylogenetic tree was constructed using whole-genome SNPs, with Tohama I (NC_002929.2) serving as the reference strain. The SNPs for BP-L1 to BP-L4 were detailed in Supplementary Table 3. The analysis also included multiple allelic types and macrolide resistance mutations, such as the 23sRNA A2047G. The phylogenetic tree identified two main lineages characterized by *ptxP/fhaB/bscI* allele profiles and the A2047G mutation. The *ptxP1* cluster primarily consists of Chinese isolates (32 out of 35 strains), including BP-L2/L3 strains with the A2047G mutation and *fhaB3* alleles. The *ptxP3* cluster includes both Chinese and Western strains, with BP-L1/L4 strains featuring *bscI3* alleles. The copy numbers of IS1002, IS481, and IS1663 in strains BP-L1 to BP-L4, CS, and the reference strain Tohama I were compared. All four clinical isolates exhibited nearly identical insertion sequence (IS) copy numbers (249 to 253 copies for IS481, 6 to 7 copies for IS1663, and 6 copies for IS1002), whereas CS and Tohama I had only 236 and 230 copies of IS481 in their genomes, respectively (see Supplementary Table 4 for details). In addition, IS481 showed consistent insertion in the gene coding regions and promoters of the four isolates. However, no insertion occurred in the known antigen gene coding regions or promoters. The specific insertion positions and functional annotation results are shown in Supplementary Table 5. More importantly, the PRN coding sequence of the BP-L2 strain was only 1,629 bp. It exhibited a substantial base deletion spanning positions 52 to 1156, corresponding to the deletion of amino acids at positions 18 to 391. The deleted segment is situated within the region encoding the P.69 protein, specifically the PRN antigen. The N-terminal signal sequence and the C-terminal self-transport element P.30 remained intact and matched the reference strain (Junker et al., 2006). Based on the sequencing results, the PRN protein encoded by BP-L2 comprises 174 amino acids, with a theoretical monomer molecular weight of approximately 19 kDa. The PRN sequence of BP-L2 is presented in Supplementary Material 1.

BP-L2 expresses partial PRN antigen

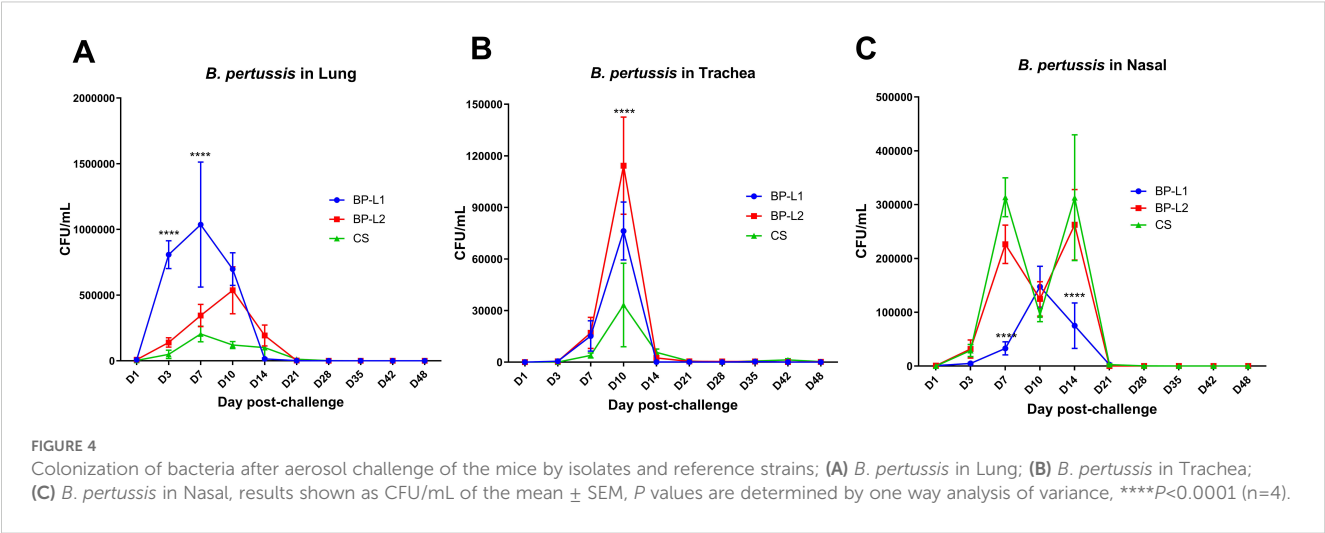
The samples of CS, BP-L1, and BP-L2 containing PRN were analyzed using SDS-PAGE and western blotting (Supplementary Figure 1). Consistent results were found for BP-L1 and CS, while the PRN components of BP-L2 formed complex bands, with the main band located at approximately 72 kDa. The N-terminal amino acid sequencing results (Figure 2) were consistent with the amino acid sequence at positions 65–83 in the PRN coding region of BP-L2 (score 490.7), demonstrating that BP-L2 was capable of expressing a partial PRN antigen.

To confirm the expression of the PRN antigen, we determined serum IgG antibody levels 6 weeks after the challenge (Figure 3). The geometric mean of antibody titers was 476 for BP-L1, 141 for BP-L2, and 673 for CS, respectively. Although the PRN antibody levels of the BP-L2 group were lower than those in the other two groups, the differences were not significant ($P > 0.05$). The results

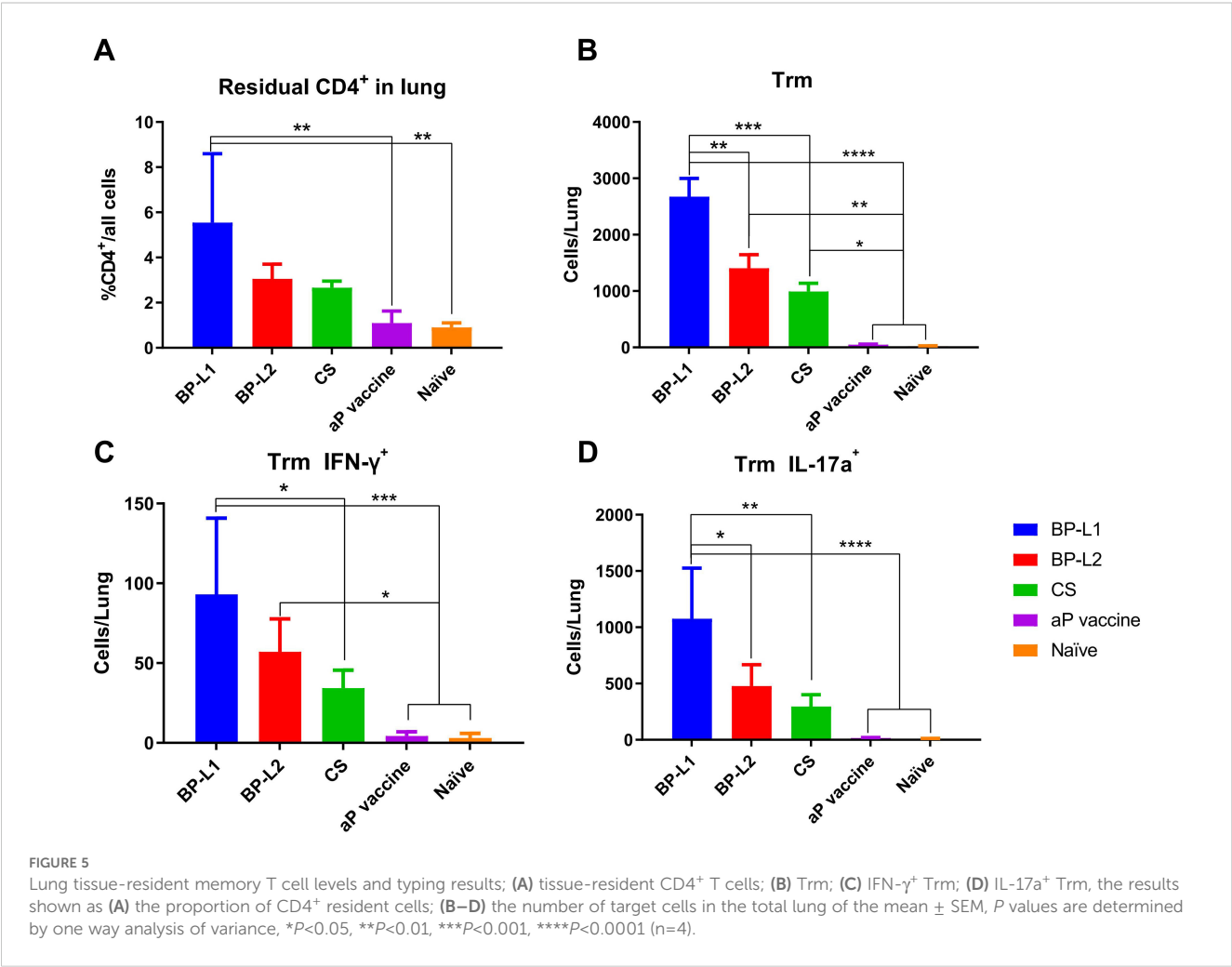


indicated that a partial PRN was expressed and that some degree of antibody response was induced during BP-L2 infection.

The binding affinities of the antiserum to the PRN of the CS, BP-L1, and BP-L2 strains were 8.8193 nM, 9.0359 nM, and 9.0448 nM by Kd values, respectively. Since Kd values and binding affinities are inversely correlated, the antigenicity ranking of PRN is CS, BP-L1, BP-L2. However, they are very similar according to the Kd values.



and the lowest in BP-L1 (Supplementary Figure 5). For the *in vivo* study, total RNA of *B. pertussis* was isolated from infected mice and transcriptome sequencing analysis was conducted (Figure 7). Overall, an average of 155,463 2-by 150-bp reads from *in vivo* samples mapped to the reference genome, resulting in 1% of the reads corresponding to the pathogen. These mapped reads cover an average of 63% of the reference genome sequences (Supplementary Table 6). Subsequent differential analysis of the BP-L1, BP-L2 and CS strains revealed only



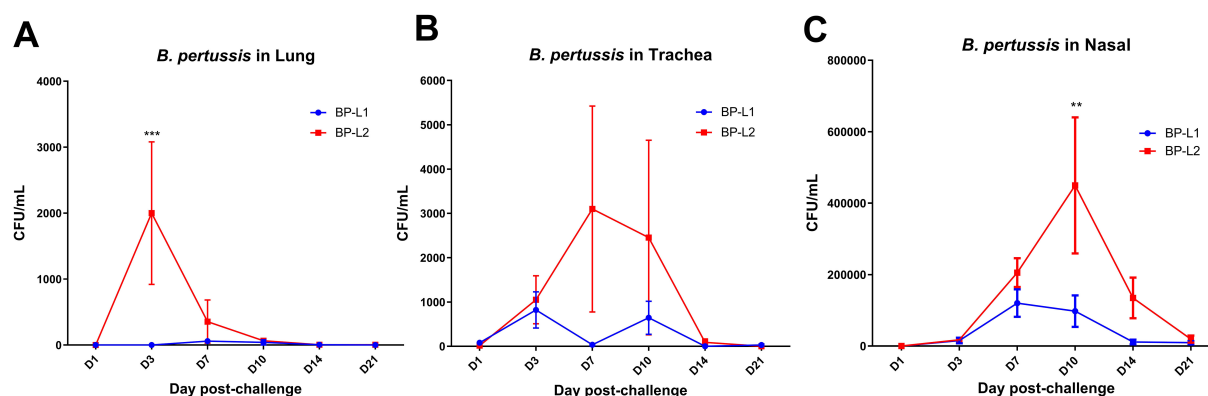


FIGURE 6

Colonization of *B. pertussis* in various regions of aP-immunized mice post-infection; (A) *B. pertussis* in Lung; (B) *B. pertussis* in Trachea; (C) *B. pertussis* in Nasal, results shown as CFU/mL of the mean $\pm$ SEM, *P* values are determined by one way analysis of variance, ***P*<0.01, ****P*<0.001 (*n*=4).

104 gene expression differences across all comparisons. Among these, expressions of *relA* (log2FC = 2.1, FDR *P*-value = 0.019), *fim3* (log2FC = 5.7, FDR *P*-value = 6.02E-28) and *sodA* (log2FC = 2.4, FDR *P*-value = 8.61E-06) in BP-L2 were significantly upregulated compared to BP-L1. All the above genes are involved in the bacterial stringent response (Sugisaki et al., 2013). For the *ptxP3* strain BP-L1, the gene *bipA* is upregulated *in vivo* as a major component of biofilms, with a log2 fold change of 1.8 and an FDR *P*-value of 0.027 when compared to BP-L2. However, no genes associated with the PT antigen were found to be significantly highly expressed during *in vivo* infection. Subsequently, we reduced the fold change analysis parameter to 1.5, yet the conclusion remained unchanged. The *in vivo* RNA-seq results for gene expression differences in all comparisons are presented in Supplementary Table 7.

The results of *in vitro* transcriptome sequencing indicated that genes associated with PT and adenylate cyclase toxin were downregulated in BP-L1 and upregulated in BP-L2. This variation

may be associated with the differential expression of the virulence regulatory gene *bvgA/S* (Chen and Stibitz, 2019) (Supplementary Figure 6). The *sodB* was significantly downregulated in BP-L1 (log2FC = -0.76, FDR *P*-value = 4.37E-11) and BP-L2 (log2FC = -0.60, FDR *P*-value = 8.13E-08) compared to CS, which was associated with antioxidant stress (DeShazer et al., 1994). The *in vitro* RNA-seq results for gene expression differences in all comparisons are shown in Supplementary Table 8.

BP-L2 and BP-L3 have higher paraquat tolerance than BP-L1, BP-L4 and CS strains

Because of the increased *sodA* expression, we predicted that BP-L2 and even BP-L3 strains of the same lineage would exhibit greater tolerance to superoxide effects. To compare the ability of different clinical strains to counter oxidative stress, we assessed their

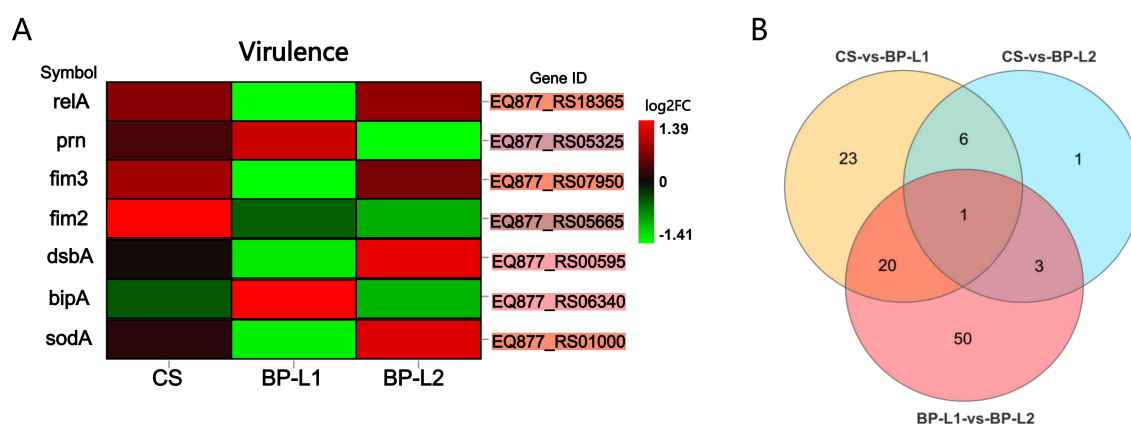


FIGURE 7

Gene expression levels of pertussis-infected strains *in vivo*; (A) Heatmap of virulence-related gene expression levels in DEGs. The background color of the 'Gene ID' represents the location in the Venn diagram; (B) Venn diagram of differentially expressed genes between the BP-L1, BP-L2 and CS group. Details of the differentially expressed genes is provided in Supplementary Table 4 marked in the corresponding color. [fold change > 2, *Q* value < 0.05, corrected by the Bonferroni test] (*n*=4).

sensitivities to paraquat, which induces oxidative stress by generating O_2^- . For this, CS and BP-L1 to BP-L4 cells were exposed to 50 mM paraquat for 30 minutes. The CFU of the bacteria before and after paraquat treatment were then counted on R-L agar plates, and the results were presented as the calculated proportion of surviving bacteria. The results indicated that the paraquat challenge caused a significant decrease (approximately 30%) in the viable counts of BP-L1, BP-L4, and CS strains, whereas the viability of BP-L2 and BP-L3 was only slightly impaired (approximately 1%) (Figure 8). These outcomes demonstrate that BP-L2 has a higher tolerance to oxidative stress ($P < 0.001$), which may be attributed to its elevated superoxide dismutases (SODs) activity. Moreover, the similar tolerance exhibited by BP-L3 within the same *ptxP1-ptxA1-fhaB3* lineage suggests that this characteristic may be lineage-specific.

Additionally, as the major component of the biofilm, *bipA* was found to have increased expression in BP-L1 infections *in vivo*. We compared the biofilm formation ability of all clinical strains of the aforementioned species using the crystal violet method, and only observed a statistical difference between BP-L2 and BP-L3 (Supplementary Figure 7). However, there was no significant difference in biofilm counts between the other strains, including BP-L1 ($P = 0.51$ compared to BP-L2).

Discussion

The resurgence of *B. pertussis*, characterized by various epidemic strains exhibiting antigenic drift and deficiencies, has facilitated the transmission of the bacterium. This study involved active monitoring

cases. From these, four clinical strains, designated BP-L1 to BP-L4, were successfully isolated. Notably, the partial PRN-deficient strain BP-L2 was reported for the first time, demonstrating a unique colonization advantage in the tracheas of naïve mice and a fitness advantage in immunized mice. *In vivo* RNA-seq results indicated that the high expression of genes associated with the stringent response and antioxidant stress during infection might contribute to BP-L2's colonization in the trachea. In line with these findings, strains BP-L2 and BP-L3, from the same lineage, exhibited significantly higher tolerance to oxidative stress, with notably elevated SODs activity. Furthermore, strain BP-L1 displayed a pronounced advantage in lung colonization and overexpressed *bipA* *in vivo*, although its biofilm formation was not enhanced.

Although the allele profiles correspond with Western strains, the *ptxP3* strains BP-L1/L4 formed a distinct clade with Chinese isolates in 2019. This suggests that the *ptxP3* strains isolated in this study may have originated from epidemic strains already prevalent in China. Furthermore, all strains isolated after 2020 in the phylogenetic tree were *bscI3* strains, including *ptxP3/fhaB1/bscI3* strains carrying macrolide resistance mutations, aligning with a recent report from China (Fu et al., 2024). This indicates that attention should be given to the macrolide resistance and *bscI* typing of *ptxP3* strains and the extent of their dissemination in China. The *ptxP3* allele has been the most frequently isolated allele in recent pertussis outbreaks (Cai et al., 2023). Mooi et al. (2009) showed by ELISAs that the PT expression of the *ptxP3* strains was modestly greater (1.62-fold) than that of the *ptxP1* strains in Bordet-Gengou media. In mice, PT plays a central role in immune suppression, facilitating early lung colonization and inhibiting neutrophil recruitment to sites of infection (Kirimaneswara et al., 2005). Consistent with mentioned studies,

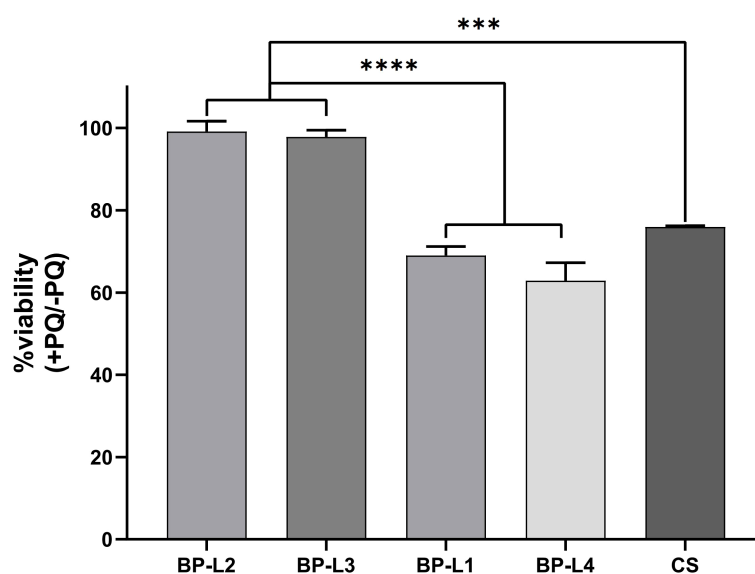


FIGURE 8

Tolerance of clinical strains BP-L1 to L4 and CS strains to paraquat. The results are calculated as percent bacterial survival after the paraquat challenge. Results are shown as mean $\pm$ SEM ($n=3$). *** $P < 0.001$ vs. CS, **** $P < 0.0001$ vs. BP-L1 and BP-L4.

and strain isolation of *B. pertussis*, screening 158 clinically suspected

we observed that the *ptxP3* strain BP-L1 had a significantly greater ability to colonize the lungs of naïve mice.

However, our *in vivo* results suggest that the colonization ability of BP-L1 in the lungs may not be directly related to the elevated PT expression, indicating that the pulmonary colonization ability of the BP-L1 strain could be associated with other mutations. Bart et al. (2010) hypothesized that *ptxP3* might be a “hitchhiker” mutation, resulting from advantageous mutations selected elsewhere in the genome, and subsequent research supports this hypothesis (Schmidtke et al., 2012). King et al. (2013) reported that *ptxP3* strains represented a lineage with a fitness advantage over other strains. In fact, previous study has also reported that no significant differences in the PT expression levels between *ptxP3* and *ptxP1* strains in THJIS medium (Luu et al., 2018), suggesting that *ptxP3* strains may represent a lineage with a series of other mutations in the genome. Our study reinforces this perspective from an *in vivo* viewpoint.

BipA, the major antigen of biofilms, has been found to be significantly upregulated during BP-L1 infection in our study. Research has indicated that biofilm formation involving *bipA* can enhance early colonization of the lungs (de Gouw et al., 2014; Hiramatsu et al., 2016). However, we verified that the biofilm formation capacity of BP-L1 was not significantly increased in the SS liquid medium. Previous studies have reported that *bipA* is expressed maximally under the Bvg-intermediate (Bvg i) phase and was also the first identified Bvg i phase gene (Williams et al., 2005). Indeed, the *in vitro* Bvg phases were defined based on specific culture conditions (Hoo et al., 2014), which may explain why the biofilm verification results are inconsistent with the *bipA* expression *in vivo*. Alternatively, biofilm formation may not be responsible for the colonization advantage of BP-L1 in the lungs, which necessitates further experimental confirmation. Nonetheless, a significant Trm response induced by BP-L1 was observed at 9 weeks post-aerosol challenge. As a significantly upregulated antigen during BP-L1 infection, *bipA* may still be a candidate antigen for enhancing the immune persistence of an aP vaccine.

RN-deficient strains have been consistently reported in countries with high vaccine coverages (Otsuka et al., 2012; Pawloski et al., 2014). Nao Otsuka found that PRN-deficient strains exhibited greater growth advantages *in vitro* (Otsuka et al., 2012), which helps maintain transmissibility between hosts. A retrospective study (Bodilis and Guiso, 2013) showed that a longer interval between the onset of pertussis symptoms and hospitalization was observed in infants infected with PRN-deficient strains, indicating an increased chance of transmission. In addition, Marjolein van Gent et al. (Salaün et al., 2003) demonstrated that PRN deletion significantly weakened lung and tracheal colonization through the PRN knockout strain. However, there appears to be no major loss of *in vitro* or *in vivo* fitness in the natural PRN-deficient strain, which raises the question of whether the contributions of PRN to pathogenesis might be redundant or complementary functions. Unlike the currently widely isolated PRN-deficient strains (Ma et al., 2021), BP-L2 was able to express the partial PRN antigen. Whole genome sequencing showed that the PRN coding region of BP-L2 was missing from amino acids 18 to 391 and the theoretical molecular weight was about

19kDa. The missing region contains the RGD sequence, which is crucial for PRN's adhesive role, and the highly variable region 1 (Junker et al., 2006). However, the N-terminal signal sequence and C-terminal transport element P.30 remained intact and identical to the reference strain. Consequently, we hypothesize that this segment of the PRN antigen can still be expressed and transported to the outer membrane. Subsequent ELISA, binding affinity tests, and LC-MS/MS analyses confirmed the expression of the partial PRN. We observed a distinct respiratory colonization advantage for BP-L2, as well as characteristics of immune escape. Thus, this pattern of partial expression may represent an adaptive evolution that maximally retains the ability to infect, highlighting the significant role of the missing part in immune protection (the RGD sequence) or the crucial role of the remaining part in the colonization and infection of *B. pertussis* (Hijnen et al., 2004).

Compared to conventional RNA sequencing, *in vivo* RNA-seq revealed a remarkably limited number of DEGs, highlighting the association between the colonization advantage and transcriptional regulation in this study. The *in vivo* expression level of PRN in BP-L2 was significantly lower than that of the other two strains. However, whether this is related to the partial deletion of the PRN coding region remains to be further studied. During BP-L2 infection *in vivo*, the *relA* and *sodA* genes were significantly overexpressed, which was related to the stringent response and antioxidant stress (Martins et al., 2018). As the major synthetase of the stringent response, *relA* plays important roles in the regulation of survival during host invasion (Pacios et al., 2020). The stringent response resists intracellular oxidative stress by regulating the production of the antioxidant enzyme *soda* (Martins et al., 2018). Superoxide radicals are the main source of bacterial oxidative stress within the host and are the primary mechanism of sterilization for some antibiotics and macrophages. SODs can rapidly eliminate superoxide radicals, which is crucial for the long-term colonization of pertussis (Nguyen et al., 2011; Schatzman and Culotta, 2018). Then, we simulated *in vivo* oxidative stress using paraquat and observed elevated SOD activity in BP-L2, which could contribute to the survival of *B. pertussis* within macrophages (Mitchell et al., 2016) and provide an advantage in tracheal colonization for BP-L2. More importantly, the same SODs activity shown by BP-L3 in the same *ptxP1-ptxA1-fhaB3* lineage suggests that this character may be lineage specific. The *fhaB3* allele has been recently identified exclusively in Chinese *ptxP1* isolates, and these strains exhibit a higher mutation rate compared to the global *ptxP1-ptxA1 B. pertussis* population (Xu et al., 2019). Considering the potential impact of increased tracheal colonization on the transmission capacity of *B. pertussis*, these findings suggest that enhanced surveillance of BP-L2 and other *ptxP1-ptxA1-fhaB3* strains is warranted. Additionally, the use of a stringent response inhibitor (Léger et al., 2021) should be contemplated for the treatment and prevention of pertussis infection.

Regarding the other *in vivo* RNA-seq results for BP-L2, there were 127 SNP differences distinguishing BP-L1 from BP-L2 (Supplementary Table 9). However, no related SNPs were identified in the mentioned genes such as *bipA*, *bvgA/S*, *sodA/B*, *fim2/3*, or *relA* between BP-L1 and

BP-L2. The Fim serotype for BP-L1 is Fim2/3, and for BP-L2, it is Fim3, thus accounting for the observed differences. Gene rearrangement is also a significant mode of adaptive evolution in pertussis. The published results support the expected correlation between IS elements and the observation of rearrangements in *B. pertussis* (Luu et al., 2018). This study noted similar IS counts in four clinical strains, and collinearity analysis revealed comparable gene rearrangements in BP-L1 and BP-L2 (Supplementary Figure 8). In contrast, the reference strains CS and Tohama I exhibited lower amounts of IS and different rearrangements, suggesting that these clinical strains may have a higher frequency of gene rearrangement.

The aP vaccine elicits a robust antibody response against PRN, which is crucial for complement-mediated killing of *B. pertussis* (Lesne et al., 2020). Consequently, PRN-deficient strains have been demonstrated to exhibit enhanced fitness in aP-immunized mice (Safarchi et al., 2015) and are more commonly found among *B. pertussis* isolates from vaccinated individuals (Bodilis and Guiso, 2013), indicating evidence of vaccine-driven evolution. Similarly, significant fitness of BP-L2 was observed in aP-immunized mice in this study. We observed a slight weakening in both the post-infection PRN antibody levels and binding affinities compared with other strains. By comparing with previous a study (Hijnen et al., 2004), it was also found that the residual PRN retained only 3 (epitope-16,17, and 18) out of 18 epitopes. Therefore, the adaptability in immunized hosts of BP-L2 may be the result of a combination of these factors. Furthermore, BP-L2 was isolated from a vaccinated adult patient, and family transmission was not ruled out. These results indicate the limited protection provided by the aP vaccine against current *B. pertussis* clinical strains.

However, this study has several limitations. Utilizing NSG mice enabled us to achieve sufficient levels of bacterial colonization for *in vivo* RNA-seq. Nonetheless, this approach also exposed *B. pertussis* to an immune-deficient environment lacking mature lymphocytes, functional macrophages, and dendritic cells, which could influence the activation and repression of certain virulence factors (Wong et al., 2019). Thus, this constitutes a limitation of the study. In future research, we plan to analyze the differential expression of target genes in normal infection models using RT-PCR, including *relA*, *sodA*, *bipA*, and other associated genes.

In summary, through *in vivo* functional analysis, animal modeling, and vaccine effectiveness studies on *B. pertussis* clinical strains, we first reported a partial PRN-deficient strain named BP-L2, which exhibited a distinct colonization advantage in the trachea and fitness in aP-immunized mice. The tracheal colonization advantage of the partial pertactin-deficient strain BP-L2 may be associated with the overexpression of *relA* and *sodA* during *in vivo* infection. Further experiments revealed that the SODs activity of BP-L2 was indeed higher than that of other strains, indicating higher paraquat tolerance. More importantly, the same tolerance displayed by BP-L3 within the same *ptxP1-ptxA1-fhaB3* lineage suggests that this characteristic may be lineage-specific. We propose that our results could offer a novel perspective for investigating emerging *B. pertussis* strains. According to the results of our study, despite Yunnan's proximity to Laos, Vietnam, and Myanmar in

southwestern China, the *B. pertussis* epidemic trend is consistent with that of China and most countries. These results are crucial not only for expanding our understanding of *B. pertussis* strains but also for promoting the development of new vaccine formulations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA1066958.

Ethics statement

The studies involving humans were approved by The ethics committee of the People's Hospital of the Xishuangbanna Dai Autonomous Prefecture. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal study was approved by The institutional Experimental Animal Ethics Committee of Institute of Medical Biology, Chinese Academy of Medical Science & Peking Union Medical College. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Data curation, Investigation, Funding acquisition, Writing – original draft. CW: Data curation, Investigation, Writing – review & editing. MH: Resources, Writing – review & editing. MZ: Data curation, Investigation, Writing – review & editing. JLL: Investigation, Writing – review & editing. XM: Data curation, Writing – review & editing. NG: Investigation, Writing – review & editing. QG: Investigation, Writing – review & editing. YM: Data curation, Writing – review & editing. JYL: Data curation, Investigation, Writing – review & editing. SL: Data curation, Writing – review & editing. YH: Resources, Writing – review & editing. MS: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. LS: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1547751/full#supplementary-material>

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Less is more: the lack of autoinducer-2-dependent quorum sensing promotes competitive fitness of *Escherichia coli* strain 83972

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Autoinducer-2 is a signaling molecule involved in quorum sensing in *Escherichia coli*. Quorum sensing enables coordinated behavior based on cell density and helps bacteria adapt to their environment. The *luxS* gene and the *lsr* locus are involved in the biosynthesis, transport, and intracellular phosphorylation of autoinducer-2. Disruption of autoinducer-2 biosynthesis or transport can reduce biofilm formation, chemotaxis, and the expression of genes relevant for the uropathogenicity of *E. coli*. Interestingly, most isolates of *E. coli* phylogroup B2, in which uropathogenic and other extraintestinal pathogenic strains are overrepresented, lack the *lsr* operon. We show that autoinducer-2-dependent quorum sensing is not fundamentally beneficial for efficient and prolonged urinary bladder colonization. We demonstrate that the *lsr*-negative asymptomatic bacteriuria isolate 83972 has a higher fitness than its *lsr*-complemented variant. Using transcriptome analyses, competitive growth assays, and comparisons of selected fitness properties, we show that restoration of the *lsr* operon in this strain background results in growth retardation, loss of competitiveness, and higher sensitivity to oxidative stress. Our results illustrate that the lack of autoinducer-2-dependent quorum sensing contributes to the well-known fitness and competitiveness of *E. coli* 83972, on which its effective use for bacterial interference in the urinary bladder relies. It is vital to delve deeper to fully understand the fitness and competitiveness of the ABU strain 83972 if we are to optimize its use in therapeutic colonization. The key is to unravel the underlying molecular mechanisms, thus ensuring the efficacy and safety of this treatment as an alternative to antibiotic therapy.

KEYWORDS

lsr locus, extraintestinal pathogenic *E. coli*, fitness, stress response, competitiveness

1 Introduction

Extraintestinal pathogenic *Escherichia coli* (*E. coli*) strains (ExPEC) are usually classified as facultative pathogens since they often colonize the gut without causing symptoms (Wiles et al., 2008; Köhler and Dobrindt, 2011). However, they harbor accessory traits that enable them to invade and colonize extraintestinal niches, eventually leading to symptomatic diseases (Vila et al., 2016; Sora et al., 2021). *E. coli* strains that have characteristic genetic markers that indicate the potential to cause extraintestinal disease, or that were directly isolated from infections outside the gastrointestinal system, are typically classified as ExPEC. ExPEC are usually divided into sepsis-causing (SEPEC), neonatal meningitis-associated (NMEC), and uropathogenic *E. coli* (UPEC), as well as strains causing systemic disease in animals such as avian pathogenic (APEC) or mammary pathogenic (MPEC) *E. coli* (Köhler and Dobrindt, 2011; Sora et al., 2021). Several virulence factors are described to promote extraintestinal pathogenicity in ExPEC, including adhesins, invasins, iron uptake systems, or toxins (Sora et al., 2021). Another bacterial trait that is involved in extraintestinal pathogenicity is quorum sensing (QS). Quorum sensing is a process in which bacteria sense and adapt to changing cell densities through signaling molecules called autoinducers (AI). Several AIs that are specific for certain bacterial strains or families have been described (Rutherford and Bassler, 2012). AI-2, on the other hand, serves as an interspecies signaling molecule that is produced and sensed by both Gram-positive and Gram-negative bacteria (Sun et al., 2004; Pereira et al., 2013). Even eukaryotic cells were proposed to participate in AI-2-mediated QS since it was found that *Saccharomyces cerevisiae* secretes molecules that mimic AI-2, as well as the intestinal epithelial cell (IEC) line Caco-2 in response to bacteria or a tight-junction disruption (Ismail et al., 2016; Valastyan et al., 2021). Moreover, the inflammatory interleukin IL-8 was found to be upregulated in response to external AI-2 in the IEC line HCT-8 (Zargar et al., 2015).

In bacteria, the precursor of AI-2, (S)-4,5-dihydroxy-2,3-pentanedione (DPD) (Xavier and Bassler, 2005), is produced as a byproduct of L-homocysteine synthesis by the synthase LuxS, a widely distributed and conserved enzyme that is part of the activated methyl cycle (Schauder et al., 2001; Vendeville et al., 2005). DPD is a hydrophilic molecule that is actively transported out of the bacterial cell after production (Khera et al., 2022). Outside of the cell, DPD spontaneously circularizes into either boron-containing (S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate or boron-free (R)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF), resulting in two different types of active AI-2 (Chen et al., 2002; Miller et al., 2004). Depending on the environmental status, DPD can spontaneously convert between the two active forms and thus be sensed by mixed bacterial communities since both AI-2 forms are perceived by different receptors (Zhang et al., 2020). R-THMF, from now on referred to as AI-2, is an intracellular signaling molecule (Xavier and Bassler, 2005). Different receptors for AI-2 are known, but the best-studied is LsrB. LsrB was first described in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (Taga et al., 2001) and is part of a specialized transporter encoded in the *lsr* (LuxS-regulated) locus (Taga et al., 2003). The *lsr* locus, or homologs, have been found in various bacterial species,

including *E. coli* (Pereira et al., 2009), and comprises two transcription units. The first transcription unit, *lsrRK*, encodes the kinase LsrK that phosphorylates the incorporated AI-2 and the repressor LsrR that represses transcription of both *lsr* operons (Xue et al., 2009), as well as of several other genes in *E. coli* (Li et al., 2007) and *S. Typhimurium* (Choi et al., 2012). The binding of phosphorylated AI-2 to LsrR abolishes the transcriptional repression, resulting in AI-2-dependent gene expression (Xavier and Bassler, 2005). The second transcription unit, *lsrACDBFG*, encodes structural proteins of the AI-2 transporter, i.e., LsrA, LsrC, LsrD, and LsrB as the receptor, as well as the isomerase LsrG that degrades the phosphorylated AI-2 and the thiolase LsrF that catalyzes the terminal step in processing phosphorylated AI-2 (Marques et al., 2014) (Figure 1).

Interfering with AI-2-dependent QS, either by using QS inhibitors (Helmy et al., 2022) or gene mutations and deletions, led to altered behavior and lower virulence in ExPEC strains. For example, the deletion of *luxS* has led to a significant reduction of virulence in the APEC O78:K80:H9 strain χ 7122 (Palaniyandi et al., 2013) and an attenuated virulence of the APEC strain DE17 Δ aroA (Han et al., 2015). Also, the deletion of *lsrACD* in APEC94, which leads to an impaired AI-2 uptake and decreased bacterial motility, has led to lower bacterial loads and reduced virulence in a duckling infection model (Zuo et al., 2019). Additionally, it was shown that AI-2-dependent QS is associated with hydrogen peroxide (H₂O₂) resistance in the MPEC strain DCM5 (Wang et al., 2021) and antibiotic sensitivity in APEC strain APECX40 and the MPEC isolate DCM1 (Xue et al., 2016; Yu et al., 2020). Although AI-2-dependent QS has significant effects on APEC and MPEC, little is known about its effects on the virulence and fitness traits of UPEC, which are often genetically very similar to APEC and MPEC (Rodríguez-Siek et al., 2005; Tivendale et al., 2010). UPEC are the leading cause of urinary tract infections (UTI), one of the most prevalent bacterial infections worldwide, accounting for at least 75% of all complicated and uncomplicated cases (Flores-Mireles et al., 2015). Despite several host defense mechanisms, including the physicochemical composition of urine, the expression of antimicrobial peptides, or other innate immune response mechanisms (Abraham and Miao, 2015; Loubet et al., 2020), more than 50% of all women (Medina and Castillo-Pino, 2019) and about 20% of all men (Farrell et al., 2021) suffer at least from one symptomatic UTI episode during their lifetime. This indicates that UPEC strains are well adapted to the bladder environment and can combat the host's defense strategies (Flores-Mireles et al., 2015; Mann et al., 2017).

The asymptomatic bacteriuria (ABU) *E. coli* isolate 83972 evolved from UPEC but has lost the ability to express many functional virulence factors (Zdziarski et al., 2008). This strain is well adapted to long-term bladder colonization without causing inflammation or other UTI-related symptoms (Hull et al., 1999). Additionally, *E. coli* 83972 has a better antioxidant defense than UPEC strains (Aubron et al., 2012) and can outcompete UPEC strains in human urine, amongst others, probably due to fast growth and nutritional adaptation (Ipe et al., 2016; George et al., 2024). Due to these characteristics, deliberate bladder colonization by *E. coli* 83972 has been suggested as a therapeutic approach to prevent recurring UTI by bacterial interference (Sundén et al., 2010; Köves

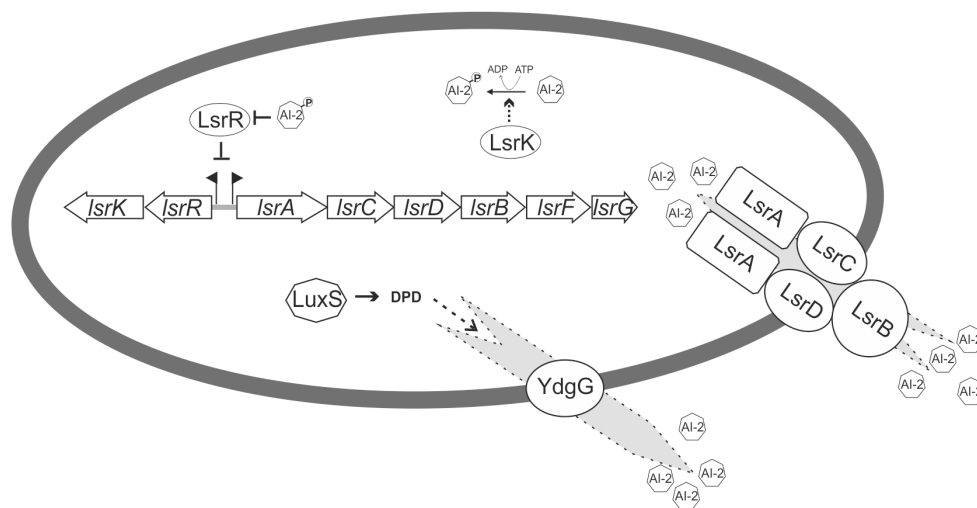


FIGURE 1

Regulatory circuit and Lsr-mediated transport and modification of AI-2 in *E. coli*. The AI-2 precursor DPD is produced by LuxS. AI-2 is then exported from the bacterial cell by YdgG. Extracellular AI-2 is imported by the ABC transporter LsrACDB. Intracellular AI-2 will be phosphorylated by the kinase LsrK and remains in the cytoplasm. The transcriptional repressor LsrR represses the expression of the *LsrACDBFG* and *lsrRK* transcriptional units by binding to their promoter region. Phosphorylated AI-2 can bind to the repressor LsrR and thereby relieve the transcriptional blockade. The subsequent expression of the LsrACDB transporter facilitates additional uptake of extracellular AI-2. Phosphorylated AI-2 is further processed by LsrG and LsrF.

et al., 2014; Wullt and Svanborg, 2016). Against the background of the increasing spread of multidrug-resistant bacteria, especially in the context of UTI, the use of antibiotics must be reduced (Cook and Wright, 2022). Bacterial interference by bladder colonization with ABU strain 83972 is an alternative way to prevent permanent colonization of the urinary bladder by symptomatic uropathogens (Wullt and Svanborg, 2016; Stork et al., 2018; Kenneally et al., 2022). Deeper insights into the importance of QS for the fitness and competitiveness of ABU strain 83972 may help to optimize the use of this bacterial strain for the deliberate therapeutic colonization of patients by uncovering underlying molecular mechanisms and thus improving the efficacy and safety of this form of treatment as an interesting alternative to antibiotic therapy. Interestingly, *E. coli* 83972 lacks the complete *lsr* locus but carries the *luxS* gene. We hypothesized that the absence of AI-2-dependent QS contributes to the strain's fitness during bladder colonization. Therefore, we integrated the full-length *lsr* locus from the *E. coli* K-12 strain MG1655 into the chromosome of *E. coli* 83972 and analyzed the transcriptome of the *lsr* complemented strain to screen for differentially expressed genes that are affected by AI-2-dependent QS and may be relevant during bladder colonization. Additionally, we tested for relevant bacterial phenotypes that may increase this strain's fitness in the urinary bladder.

2 Material and methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in [Supplementary Table S5](#). The asymptomatic bacteriuria *E. coli* isolate 83972 has been obtained as a gift from C. Svanborg (Lund, Sweden). Bacterial cultures were

either cultivated in lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or pooled human urine (four male and six female voluntary individuals, sterile filtered and mixed in a 1:1 male/female ratio (v/v)). When appropriate, antibiotics were added in the following concentrations: kanamycin (25 µg/mL), chloramphenicol (12.5 µg/mL), ampicillin (100 µg/mL), or zeocin (50 µg/mL). Bacterial strains were grown overnight at 37°C on LB agar plates (containing 1.5% agar (w/v)) with the appropriate antibiotics when needed. For overnight cultures, single colonies were picked and incubated in 2 mL of LB at 37°C and with orbital shaking at 180 rpm. *E. coli* DH5α was used as a host for plasmid construction. Genome manipulation was done by recombineering (Datsenko and Wanner, 2000). When indicated, cultures were supplemented with H₂O₂ (stabilized, Merck Millipore, Darmstadt, Germany) or synthetic DPD purchased from Rita Ventura's research group at ITQB-UNL (Oeiras, Portugal) (<https://itqb.unl.pt/research/chemistry/bioorganic-chemistry>). Synthetic DPD was synthesized as published before (Ascenso et al., 2011), and the concentration was 16.8 mM. Both chemicals were subdiluted to working concentrations using sterile ddH₂O.

Cloning methods. DNA amplification for cloning and genetic manipulation was done using the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt/Main, Germany). Colony-PCR was done using the GoTaq Green Master Mix (Promega GmbH, Walldorf, Germany). After amplification, the correct size of amplicons was verified using agarose gel electrophoresis in 1x Tris-acetate-EDTA (TAE) buffer with 1–2% (w/v) agarose. The gels were run at 110–130 V for 30–60 min. Amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) or the Wizard SV Gel and PCR Clean-Up System kit (Promega, Walldorf, Germany).

Plasmids were isolated using the NucleoSpin Plasmid Mini kit (Macherey-Nagel). Genomic DNA (gDNA) was isolated using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Purified PCR products were quantified using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Schwerte, Germany). The restriction enzymes and T4 DNA ligase used for cloning were purchased from New England Biolabs (Frankfurt/Main, Germany).

Oligonucleotides and plasmids used. All oligonucleotides and plasmids used are listed in [Supplementary Tables S6, S7](#).

Construction of pMK2. The plasmid pMK2 was constructed by integrating the *lsrA* promoter-*yfp* fusion reporter cassette coupled to *lsrR* (*lsrR:PlsrA:yfp* fusion) from the previously described *E. coli* K-12 strain MG1655 (*lsrA-G::yfp-cat*) (Keizers et al., 2022) into the low-copy number plasmid pWKS30 (Wang and Kushner, 1991). For that, the *lsrR:PlsrA:yfp* fusion was amplified from the gDNA of *E. coli* K-12 strain MG1655 (*lsrA-G::yfp-cat*) using the oligonucleotides pKD3_lsr_seq_4 and LZP50 and introduced into the *Sma*I-digested pWKS30 by using DNA T4 ligase. The correct insertion of the PCR product into pWKS30 was confirmed by plasmid preparation and subsequent Sanger sequencing.

Construction of pLS1 and pLS2. The low-copy-number plasmids pLS1 and pLS2 were constructed by integrating the *Pdps-cfp* (*cfp* under the control of the stationary phase-dependent *dps* promoter) cassette or the *Pdps-yfp* (*yfp* under the control of the stationary phase-dependent *dps* promoter) cassette, respectively, into pWKS30. Both cassettes were amplified using the oligonucleotides bla-TEM-r and MC_185 and pPS1 (*Pdps-cfp*) or pPS2 (*Pdps-yfp*) (Schiller et al., 2021) as templates. Amplicons and pWKS30 were cut with *Kpn*I and *Sma*I. All products were purified, and the digested *Pdps-cfp* cassette was introduced into digested pWKS30, resulting in pLS1. The digested *Pdps-yfp* cassette was introduced into digested pWKS30, resulting in pLS2. Ligation was done using DNA T4 ligase. Correct plasmids were confirmed by fluorescence microscopy.

Construction of *E. coli* strains 83972 $\Delta luxS$ and 83972 $\Delta ybhC$. For the construction of *E. coli* 83972 $\Delta luxS$, the *luxS* gene was replaced by a zeocin resistance cassette (*bleR*) that was amplified using the oligonucleotides Rec_BleoR_fwd and Rec_BleoR_rev and pEM7/Zeo as a template. Replacement was confirmed by colony PCR using the oligonucleotides CFT073_ΔluxS_CP_fwd and CFT073_ΔluxS_CP_rev and Sanger sequencing of the purified amplicon. For the construction of *E. coli* strain 83972 $\Delta ybhC$, the *ybhC* gene was replaced by a chloramphenicol resistance cassette (*cat*) that was amplified using the oligonucleotides del_ybhC_fwd and del_ybhC_rev and pLP2 (Peng et al., 2022) as a template. Replacement was confirmed by colony PCR using the oligonucleotides wt_PybhC and LZP18 and Sanger sequencing of the purified amplicon.

Construction of *E. coli* strains 83972 *attB::lsr* and 83972 $\Delta luxS$ *attB::lsr*. First, the plasmid pWKS30_LSR was generated. The chloramphenicol resistance cassette (*cat*) was introduced upstream of the *lsr* locus in the chromosome of *E. coli* strain MG1655 by recombineering (Datsenko and Wanner, 2000). The resistance cassette was amplified using the oligonucleotides CBL_fwd and CBL_rev and pMB54 (Berger et al., 2016) as a

template. The correct chromosomal insertion was confirmed by colony PCR using the oligonucleotides LKRS_fwd and pMB54_lsrRK_CP1_rev. In the same way, the kanamycin resistance cassette (*aph*) was chromosomally inserted downstream of the *lsr* locus in the resulting *E. coli* strain MG1655 *cat::lsr*. The *aph* resistance cassette was amplified using the oligonucleotides RBL_fwd and RBL_rev and pKD4 as a template. The correct chromosomal insertion was confirmed by colony PCR using the oligonucleotides pKD3_lsr_seq_12 and LZP50. The full-length *lsr* locus, flanked by *cat* and *aph*, was amplified from gDNA of the resulting *E. coli* strain MG1655 *cat::lsr_aph* using the oligonucleotides LKRS_fwd and LZP50, and cloned into the *Sma*I-digested pWKS30 by using DNA T4 ligase. The relevant parts of the resulting plasmid pWKS30_LSR were afterward confirmed by Sanger sequencing. The complete *lsr* determinant was then inserted by recombineering (Datsenko and Wanner, 2000) next to the chromosomal attachment site of the bacteriophage λ (*attB*) in either *E. coli* 83972 or strain 83972 $\Delta luxS$. For this, the *lsr* locus flanked by *cat* and *aph* (*cat::lsr_aph*) was amplified using the oligonucleotides pWKS30_attB_fwd and pWKS30_attB_rev and pWKS30_LSR as a template and introduced next to the λ *attB* site of the strains 83972 and 83972 $\Delta luxS$, respectively. The correct chromosomal insertion was confirmed by colony PCR using the oligonucleotides CFT073_lsrRK_YFP_CP1_fwd and pKD3_lsr_seq_12 or CFT073_lsrRK_YFP_CP1_fwd and CFT073_lsrRK_YFP_CP2_rev and pKD3_lsr_seq_13. Afterward, gDNA was isolated, and the *cat::lsr_aph* region was amplified using the primer pair CFT073_lsrRK_YFP_CP1_fwd and CFT073_lsrRK_YFP_CP2_rev before the correctness of the DNA sequence was also verified by Sanger amplicon sequencing.

RNA isolation. Total RNA was isolated as previously described (Wallenstein et al., 2020) with the following differences: Overnight cultures of *E. coli* strains 83972 and 83972 *attB::lsr* were diluted to a final optical density OD₆₀₀ = 0.02 in 200 mL LB. At indicated time points, 70 mL of culture (lag phase), 5 mL of culture (exponential phase), or 2 mL of culture (stationary phase) were added to the 0.2x volume of pre-chilled stop solution, and the bacterial pellets were directly lysed. Confirmation of complete gDNA removal was done by qPCR. RNA was extracted from three biological replicates for each strain.

RNAseq and data analysis. Strand-specific cDNA libraries were prepared from the isolated RNA and sequenced (Illumina NextSeq 500, 1 x 75 bp single reads) by vertis Biotechnology AG (Freising, Germany). The obtained raw reads were analyzed as follows: Quality control using FastQC and MultiQC (Bioinformatics; Ewels et al., 2016), adaptor trimming using Cutadapt (Martin, 2011) with subsequent quality control, alignment of reads using burrows-wheeler alignment (Li and Durbin, 2009) to the genome of *E. coli* 83972 *attB::lsr* with a subsequent strandedness check using RSeQC (Wang et al., 2012) and quality control, count reading using featureCounts (Liao et al., 2014) and a subsequent differentially expressed gene analysis using DESeq2 (Love et al., 2014) in R. Conversion of formats was done using AGAT (Dainat, 2022). The subsequent data analysis was done in R. The Venn diagram (Figure 2A) was made using venn.diagram (Chen, 2022). The

heatmaps (Figures 2B–D) were made using pheatmap (Kolde, 2019). Differentially expressed genes were clustered using dist() and hclust(), and biological replicates were clustered using pheatmap (cluster_cols = T) with the complete linkage method for hierarchical clustering. The scatterplots (Supplementary Figure S2) were computed using pairs.panels() from the package psych (Revelle, 2023).

Growth analysis and co-culture assays. For growth analysis, overnight cultures of the bacterial strains were diluted to a final optical density (OD₆₀₀) of 0.01 in 1 mL of fresh medium with antibiotics when appropriate. 200 µL of freshly diluted cultures were added into one well of a 96-well plate (Thermo Fisher Scientific, Schwerte, Germany) in duplicates for each biological replicate. For the subsequent growth analysis, either an Infinite M NANO+ or an Infinite F200 microplate reader (both from TECAN, Männedorf, Switzerland) was used. The optical density was either measured at 595 nm (± 9 nm) (M NANO+) or 595 nm (± 10 nm) (F200). Signals were measured in ten-minute intervals. The resulting growth curves were analyzed using AMiGA (Analysis of Microbial Growth Assay (Midani et al., 2021)). For the lag phase length analysis, the data was not log-transformed. The co-culture assays were performed as previously described (Keizers et al., 2022). Briefly, overnight cultures of the strains to be analyzed were mixed in a 1:1 ratio with the *E. coli* K-12 reporter strain MG1655 $\Delta luxS$ attB::PlsA-yfp to a final optical density (OD₆₀₀) of 0.02. Growth analysis was performed as described above, and the fluorescence signal was either measured using an excitation wavelength of 514 nm (± 9 nm) and an emission wavelength of 550 nm (± 20 nm) (M NANO+) or using an excitation wavelength of 485 nm (± 20 nm) and an emission wavelength of 535 nm (± 25 nm) (F200).

Competition assays. For the competition assays, overnight cultures of *E. coli* strains 83972 (pLS2) and 83972 attB::lsr (pLS1) were mixed in a 1:1 ratio to a final optical density (OD₆₀₀) of 0.02 in 25 mL of fresh medium supplemented with ampicillin. The cultures were grown at 37°C with orbital shaking at 180 rpm. After 3 h of growth, the cultures were subdiluted into 25 mL of fresh medium containing ampicillin with an appropriate dilution factor (1:200 for LB and 1:10 for pooled human urine). After subdilution, the cultures were grown for another 3 h. The subdilution procedure was repeated three times with a total assay time of 12 h. For the long-term competition, the cultures were grown at 37°C with orbital shaking at 180 rpm for 72 h. Directly after mixing and at the indicated time points, aliquots were taken from the cultures and microscopically analyzed using a Leica inverted microscope DMi8 with an attached camera at 400 x magnification. For each aliquot, ten microscopic pictures were taken with three channels each – Differential interference contrast (DIC), cyan fluorescent protein CFP (excitation 436 nm (± 20 nm); dichroic mirror 455 nm; emission 480 nm (± 40 nm) and fluorescein isothiocyanate (FITC) (excitation 480 nm (± 40 nm); dichroic mirror 505 nm; emission 527 nm (± 30 nm). Next, the number of bacteria per picture was quantified in the CFP channel and the FITC channel using an ImageJ script. The ratio of bacterial numbers in the FITC

channel compared to the total bacterial numbers in both the FITC and CFP channels was calculated for each of the ten pictures, resulting in one mean ratio value with a standard deviation for each time point and biological replicate.

H₂O₂ resistance assay. For the H₂O₂ resistance assay, overnight cultures of the bacterial strains were diluted to a final optical density (OD₆₀₀) of 0.01 in 1 mL of fresh medium. 190 µL of freshly diluted cultures were added into one well of a 96-well plate (Thermo Fisher Scientific) in duplicates for each biological replicate. After adding cultures, 10 µL of H₂O₂ diluted in ddH₂O was added into each well, resulting in the indicated final H₂O₂ concentrations. The growth was analyzed directly after the addition using an Infinite M NANO+ plate reader (TECAN) or an Infinite F200 (TECAN). The optical density was either measured at 595 nm (± 9 nm) (M NANO+) or 595 nm (± 10 nm) (F200). Signals were measured in ten-minute intervals. The resulting growth curves were analyzed using AMiGA (Analysis of Microbial Growth Assay (Midani et al., 2021)). For the lag phase length analysis, the data was not log-transformed.

Galleria mellonella larvae feeding assay. *Galleria mellonella* larvae were purchased from Fauna Topics Zoobedarf Zucht und Handels GmbH (Marbach am Neckar, Germany) and reared on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% beeswax, 11% honey, and 11% glycerin) as previously described (Mukherjee et al., 2020). To evaluate the fitness of the *E. coli* wild type strain 83972 and its *lsr*-complemented mutant in the larval digestive tract, overnight cultures (OD₆₀₀ = 1, grown in LB) of both strains were mixed in a 1:1 ratio and force-fed to sixth-instar larvae (weighing approximately 250–300 mg) (Lange et al., 2019). A 10-µL aliquot of this bacterial suspension was gently introduced into the larval mouth using 1-mL disposable syringes fitted with 0.4 x 20-mm needles mounted on a microapplicator. Control larvae received an equivalent volume (10 µL) of sterile LB. Following force-feeding, larvae were incubated at 37°C for 24 hours without food. All larvae survived the incubation period and were subsequently flash-frozen in liquid nitrogen, ground into a fine powder, and homogenized in LB. The homogenates were then plated onto LB agar, and colony-forming units (CFUs) were counted following a 24-hour incubation at 37°C. The survival of the wild type and *lsr*-complemented mutant strains was analyzed by comparing their initial inoculation ratio to the ratio recovered from larvae after 24 hours of incubation.

Detection of the *lsr* determinant in *E. coli* genomes. To screen for the presence and conservation of the *lsr* determinant in *E. coli*, all published *E. coli* genomes that were available at the time point of the analysis were downloaded from NCBI Assembly (Kitts et al., 2016) (time point of download: 15.03.2023) in FASTA format (Status: latest RefSeq). In total, 32,594 genomes were downloaded. Next, the phylogroup was determined using ClermonTyping (Beghain et al., 2018) for each genome. The analysis was continued with 32,404 genomes belonging to the phylogroups A, B1, B2, C, D, E, F, and G. Next, each genome was analyzed using the basic local alignment search tool (BLAST+ (Camacho et al., 2009)) to screen for the nucleotide sequence (blastn) of the full-length *lsr*

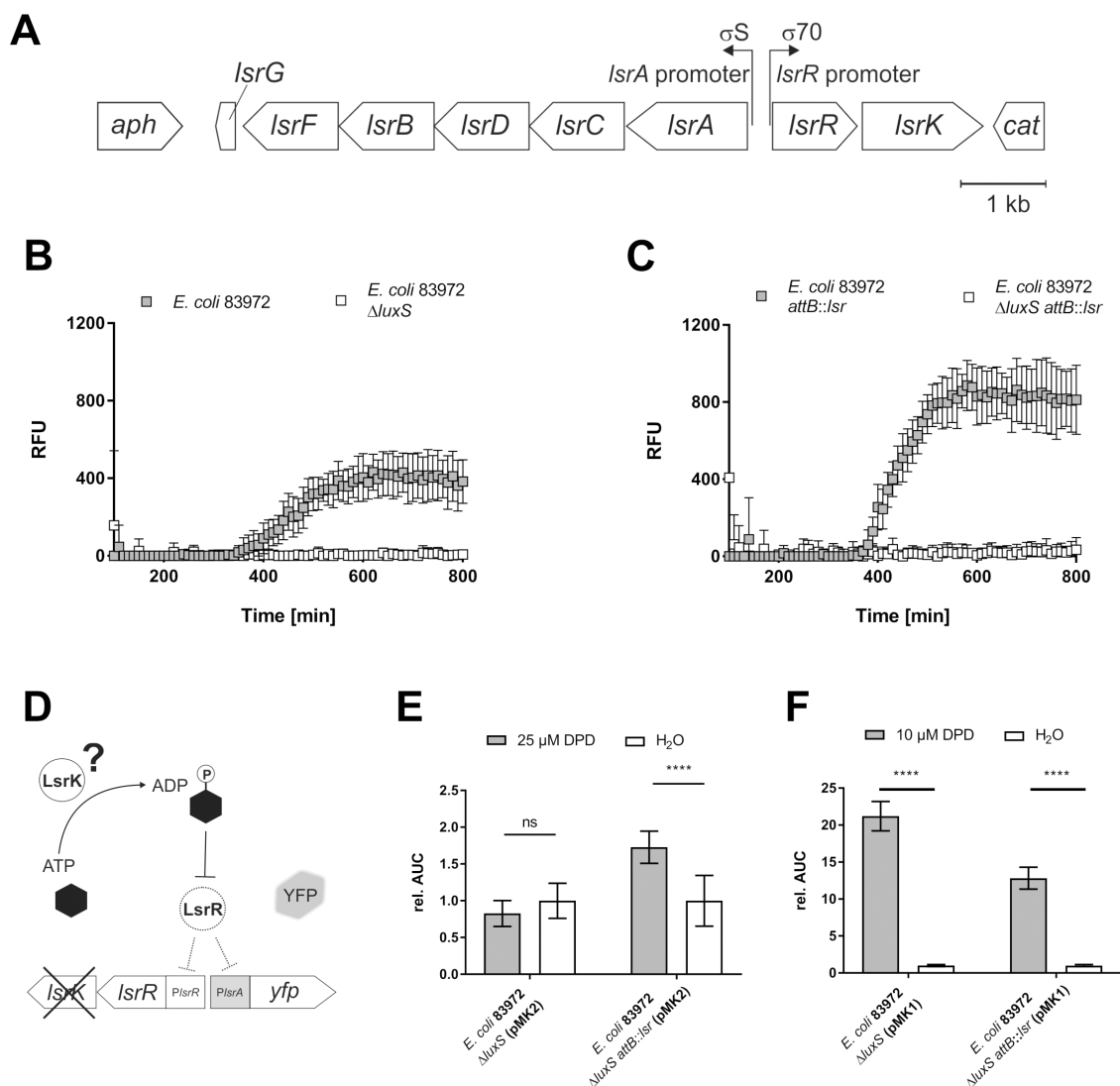


FIGURE 2

Complementation of *E. coli* 83972 with the *lsr* determinant and its impact on AI-2 synthesis, uptake and LsrK phosphorylation. The full-length *lsr* locus was introduced into *E. coli* 83972, and subsequent analyses revealed *luxS*-dependent AI-2 synthesis, the functionality of the complemented gene *lsrK* and the functionality of AI-2 uptake. (A) The *lsr* determinant flanked by two resistance cassettes (12,520 bp) was integrated into the chromosomal *attB* site of *E. coli* 83972. The *luxS*-dependent AI-2 synthesis was analyzed by fluorescence kinetics (100–800 min), depicted as RFU, during co-culture with the reporter strain *E. coli* Δ *luxS* (*attB::PlsA-yfp*) (Keizers et al., 2022). Co-cultures consisting of the reporter strain and (B) either *E. coli* 83972 (grey squares) or *E. coli* 83972 Δ *luxS* (white squares) or (C) *E. coli* 83972 *attB::lsr* (grey squares) and *E. coli* 83972 Δ *luxS attB::lsr* (white squares) in a 1:1 ratio in LB. Error bars represent the results of three biological replicates in duplicates each. (D) The functionality of the complemented gene *lsrK* was analyzed by incorporating the reporter plasmid pMK2 (comprises *lsrR* and the *PlsA-yfp* reporter module) into *E. coli* strains 83972 Δ *luxS* and 83972 Δ *luxS attB::lsr*. YFP expression depends on AI-2 availability and the functional autoinducer-2 kinase LsrK. (E) Relative AUC calculation of *PlsA* induction in *E. coli* strains 83972 Δ *luxS* (pMK2) and 83972 Δ *luxS attB::lsr* (pMK2) after the addition of 25 μ M DPD compared to water during the late-exponential growth phase, as described before (Keizers et al., 2022). Statistical analysis was performed using the ordinary two-way ANOVA with Tukey's multiple comparison test; values < 0.05 were considered statistically significant. Error bars represent the results of five biological replicates in duplicates each. (F) Relative AUC calculation of *PlsA* induction in *E. coli* strains 83972 Δ *luxS* (pMK1) and 83972 Δ *luxS attB::lsr* (pMK1) after the addition of 10 μ M DPD compared to water during the late-exponential growth phase, as described previously (Keizers et al., 2022). Statistical analysis was performed using Welch's t-test; a value < 0.05 was considered statistically significant. Error bars represent the results of three biological replicates in duplicates each (ns, not significant, **** $p < 0.0001$).

locus (*lsrRK-lsrACDBFG*), the *luxS* gene and the *tam* gene (reference sequences from the *E. coli* K-12 strain MG1655). Output possibilities were “complete” (blastn hit length equal query length), “incomplete” (blastn hit length unequal query length), “no match” (no blastn hit) or “multiple matches” (multiple blastn hits). The output “< 100 bp” is a summary of a

blastn hit length of below 100 bp and the “no match” hits. Please note that our analysis only focuses on gene length, whereas the functionality of the encoded gene product was not part of the analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v8.0.2 (San Diego, USA).

3 Results

3.1 Complementation of *E. coli* 83972 with the *lsr* locus: impact on AI-2 uptake, synthesis and secretion

We complemented *E. coli* strain 83972 with the *lsr* locus by integrating the full-length *lsr* determinant (*lsrACDBFG* and *lsrRK* operons), flanked by two resistance markers, into the chromosomal attachment site (*attB*) of the bacteriophage λ (Figure 2A). The resulting *E. coli* strain 83972 *attB::lsr*, the wild type strain 83972, as well as their isogenic *luxS* deletion mutants (83972 $\Delta luxS$ and 83972 $\Delta luxS$ *attB::lsr*), were used for further analysis. The ability of strains 83972 and 83972 *attB::lsr* to secrete AI-2 was evaluated in co-cultures with the reporter strain *E. coli* $\Delta luxS$ (*attB::PlsA-yfp*), as described previously (Keizers et al., 2022). We detected AI-2-dependent YFP-expression in the co-cultures with *E. coli* 83972 and *E. coli* 83972 *attB::lsr*, whereas we observed no YFP-expression in co-cultures with the isogenic *luxS* deletion strains. Both strains 83972 and 83972 *attB::lsr* synthesized AI-2 in a *luxS*-dependent manner and exported it, which can be sensed by the *PlsA* reporter strain in the co-culture. In the presence of the *lsr* determinant, *E. coli* 83972 can accumulate additional AI-2, leading to a stronger *lsrA* promoter induction in the reporter strain (Figures 2B, C).

To test if the *lsr*-complemented strain was capable of taking up and phosphorylating AI-2, we introduced pMK2, a derivative of the previously described reporter plasmid pMK1 that lacks *lsrK* (Keizers et al., 2022), into the strains 83972 $\Delta luxS$ and 83972 $\Delta luxS$ *attB::lsr* (Figure 2D). The functionality of LsrK was analyzed by supplementing the growth medium with 25 μ M synthetic DPD in the late logarithmic growth phase and comparing the AI-2-dependent YFP expression to a water control. There was no significant difference in YFP expression between *E. coli* 83972 $\Delta luxS$ (pMK2) supplemented with 25 μ M DPD and the water control. The expression of the AI-2 kinase LsrK in *E. coli* 83972 $\Delta luxS$ *attB::lsr* (pMK1) led to a significantly higher YFP expression when the strain was supplemented with 25 μ M DPD as compared to the water control ($p < 0.001$) (Figure 2E). To determine if the observed differences were due to an overall defect in AI-2 uptake in the *lsr*-negative 83972 wild type strain, we introduced the reporter plasmid pMK1 into *E. coli* 83972 $\Delta luxS$ and *E. coli* 83972 $\Delta luxS$ *attB::lsr*. Again, AI-2 uptake was analyzed by supplementing the medium with 10 μ M synthetic DPD in the late logarithmic growth phase and comparing the AI-2-dependent YFP expression to a water control. In both strains harboring pMK1, we detected a significantly higher YFP expression after the addition of 10 μ M DPD relative to the corresponding water controls ($p < 0.001$). This confirmed that external AI-2 can be taken up by *E. coli* 83972 in an LsrACBD-independent way (Figure 2F).

3.2 Influence of the *lsr* determinant on global gene expression in *E. coli* 83972

To analyze the effect of AI-2-dependent quorum sensing on the transcriptome, we performed an RNA-seq analysis of *E. coli* strains

83972 and 83972 *attB::lsr*. Both strains were cultivated in LB, and samples were taken in the lag phase, during mid-exponential growth (exp. phase), and during the transition to the stationary phase (stat. phase) (Supplementary Figure S1). We isolated total RNA from three biological replicates of each strain and time point for RNAseq analysis. For each library, we obtained $\sim 11.5 \times 10^6$ reads ($\pm 2 \times 10^6$ reads), from which, on average, $\sim 89\%$ ($\pm 3\text{--}5\%$) were mapped to the reference genome (*E. coli* 83972 *attB::lsr*). Data analysis was done using DESeq2 in R (Love et al., 2014), and the resulting expression data was used for further analysis (Supplementary Figure S2). The transcriptome analysis revealed 492 significantly differentially expressed genes (DEG; adjusted p -value < 0.05) in *E. coli* 83972 *attB::lsr* compared to the wild type strain 83972 in the three growth phases. A complete list of all DEGs is provided in the Supplementary Table S1. Of the 492 DEGs, 199 genes had a log2-fold change (L2FC) in expression of at least 1 (DEG (± 1 L2FC)). Among all DEG (± 1 L2FC), nine genes were differentially expressed during all three growth phases (Figure 3A). We found 60 DEG (± 1 L2FC) in the lag phase, 67 DEG (± 1 L2FC) during mid-exponential growth, and 72 DEG (± 1 L2FC) during the transition to the stationary phase (Figures 3B–D). Besides *lsrR*, *lsrK* and the two resistance cassettes *aph* and *cat* that were used to complement *E. coli* 83972 *attB::lsr*, genes related to the isoleucine/valine biosynthesis pathway, namely *ilvNBAG*, were upregulated, and the gene *ybhC*, which encodes an uncharacterized protein that was suggested to be involved in H₂O₂ resistance, was downregulated in all three growth phases. The DEG (± 1 L2FC) can be functionally clustered into four main groups, i.e., metabolism, motility/biofilm formation/adhesion, growth and stress response, and some remaining genes (Tables 1–5).

In the lag phase, mainly biosynthesis genes were upregulated. Together with the genes of the valine, leucine, and isoleucine biosynthesis pathway (*ilvNBAMDEGC*), also genes of the *nrd* operon, encoding for a ribonucleotide reductase (*nrdHIEF*) were significantly upregulated. On the contrary, several metabolic genes were downregulated. We found that genes involved in arginine uptake and metabolism (*argT*), sulfur utilization (*ssuD*), or coding for an alanine/glycine:cation symporter (*yaaJ*), as well as a mannonate dehydratase (*rspA*) were downregulated. Also, the expression of genes associated with the aminoacyl-tRNA biosynthesis of lysine (*trnK1-5*) and valine (*trnV3-5*) or with resistance against extracytoplasmic stress (*cpXP*) was downregulated. Amongst all genes that were downregulated in strain 83972 in the presence of the *lsr* determinant in the lag phase, more than 50% code for components of the flagellar assembly apparatus. In addition to the flagellar sigma factor σ^{28} -encoding gene *fliA*, the transcriptional regulator gene *fliZ*, and the chaperone gene *flgN*, nearly all flagellar genes belonging to class 2 were downregulated (*fliE*, *fliFG*, *fliI*, *fliLMNOP*, *flhBA*, *flgBCDE*, *flgGHI*, and *flgA*).

During mid-exponential growth, we predominantly found genes associated with metabolic pathways upregulated in *E. coli* 83972 *attB::lsr* relative to *E. coli* strain 83972. The encoded gene products are involved in the metabolism of D-serine (*dsdA*), glutamate (*gltI*), glycine (*gcvPH*), L-lactate (*lldD*), sorbitol (*srlD*),

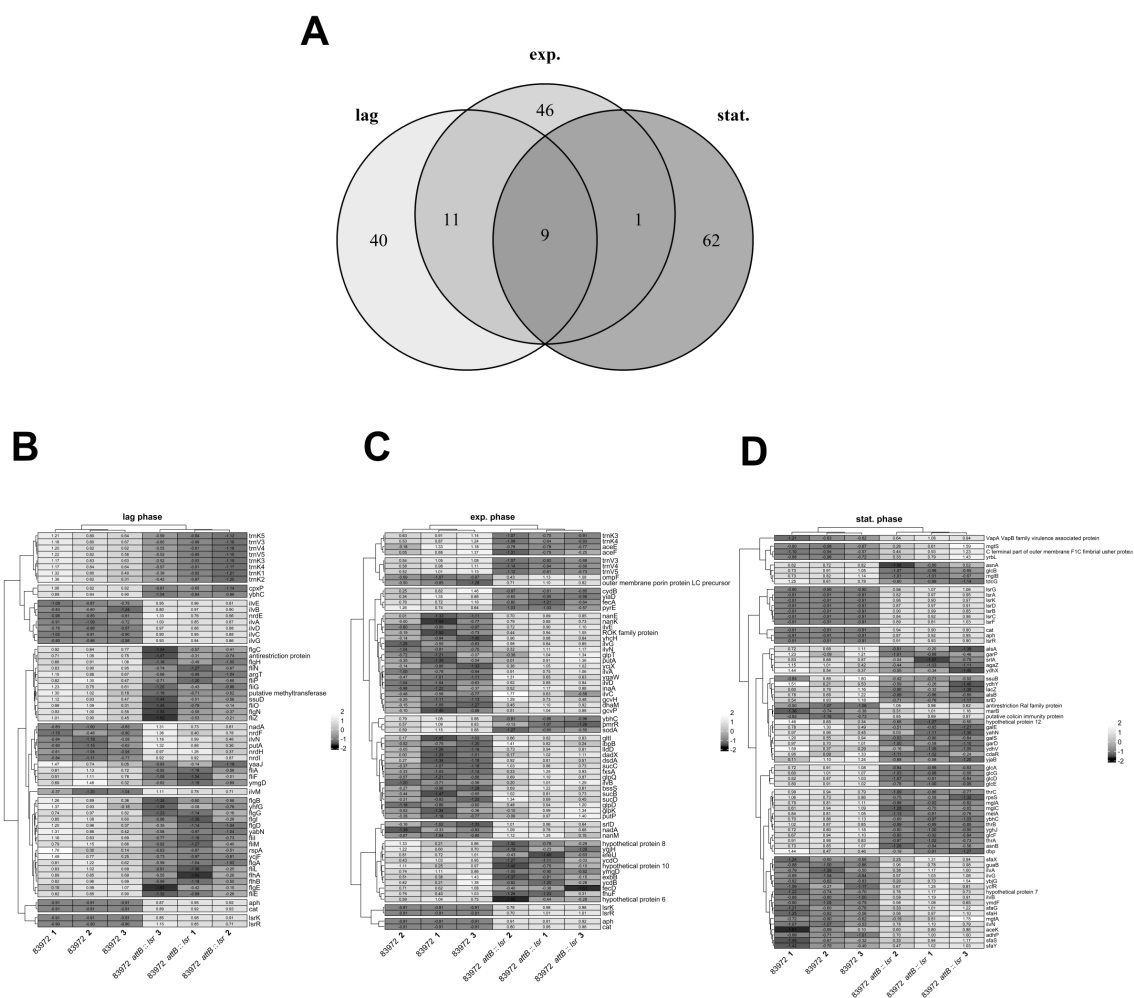


FIGURE 3

Differentially expressed genes in *E. coli* 83972 *attB::lsr* compared to its *lsr*-negative parental strain 83972. (A) The Venn diagram indicates the number of differentially expressed genes (DEG) in the lag phase (lag), mid-exponential phase (exp.) and stationary phase (stat.). The heatmaps depict the groups of genes which are differentially expressed in an *lsr*-dependent manner in (B) lag phase, (C) exp. phase and (D) stat. phase.

L-alanine (*dadX*, *ygaW*), glycerol (*glpDTQK*), proline (*putAP*), N-acetylneuraminate (*nanMKE-yhCH*), dihydroxyacetone (*dhaM*) as well as the TCA cycle (*sucBCD*). Also, three genes encoding outer membrane proteins (*ompF* and the outer membrane porin protein LC precursor) were upregulated. The genes coding for the valine, leucine, and isoleucine biosynthesis pathway (*ilvNBADEGC*) were upregulated. Interestingly, we also found upregulated genes involved in stress response, including the nitrogen starvation gene *ycjX*, the heat-shock protein gene *ibpB*, and the pH-stress response protein gene *inaA*. On the contrary, the gene cluster *aceEF*, encoding a pyruvate dehydrogenase, was downregulated during mid-exponential growth. Of all downregulated genes during mid-exponential growth, 36% are associated with iron sensing and utilization, including the genes coding for the ferric siderophore reductase (*fhuF*), the ferric chelate reductase (*yqjH*), the ferric citrate transporter genes (*fecA*) and (*fecD*), the ferrous iron permease and transport genes *efeU*, *ycdB*, and *ycdO*, the energy transducing Ton complex subunit gene *exbB* and *pmrR*, which is associated with the iron and acidic pH sensing BasSR complex.

Additionally, apart from *ybhC*, the superoxide dismutase SodA-encoding gene was downregulated. The latter two proteins have been described to protect against oxidative stress.

As expected, we observed that the genes of the *lsr* locus were highly expressed during the transition to the stationary phase. Also, the expression of valine, leucine, and isoleucine biosynthesis pathway genes (*ilvGABN*) and genes contributing to magnesium sensing and utilization were upregulated. These included the import-associated genes *mgtA* and *mgtS* and the Mg^{2+} -induced kinase gene *yrbL*. Interestingly, several genes associated with general stress response were upregulated. These included *marB*, encoding the protein MarB that reduces the transcription rate of *marA*, which codes for a pleiotropic regulator also involved in general stress response. The expression of *ycfR*, which is involved in stress response and outer membrane permeability was also upregulated. We also detected that the transcript levels of several genes (*focDFGHYX*) of the F1C fimbrial operon were significantly increased in *E. coli* 83972 *attB::lsr* in comparison to *E. coli* strain 83972. The main proportion of downregulated genes, was related to

TABLE 1 Differentially expressed genes in *E. coli* 83972 *attB::lsr* relative to *E. coli* 83972 associated with metabolism.

Gene name	log2-fold change	Pathway/function ^{82–84}
lag phase		
<i>ybhC</i>	-3.442713222	pentose and glucuronate interconversions
<i>ssuD</i>	-1.736408299	sulfur metabolism
<i>argT</i>	-1.686346996	lysine/arginine/ornithine ABC transporter
<i>yaaJ</i>	-1.090400775	alanine or glycine:cation symporter
<i>rspA</i>	-1.020974991	pentose and glucuronate interconversions
<i>putA</i>	1.245756938	alanine, aspartate and glutamate metabolism; arginine and proline metabolism
<i>ilvN</i>	1.272441162	butanoate metabolism; C5-Branched dibasic acid metabolism/valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>nadA</i>	1.292375467	nicotinate and nicotinamide metabolism
<i>ilvB</i>	1.403448526	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvA</i>	2.26260477	glycine, serine and threonine metabolism; valine, leucine and isoleucine biosynthesis
<i>ilvM</i>	2.345314152	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvD</i>	2.459224415	valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvE</i>	2.660495823	cysteine and methionine metabolism; valine, leucine and isoleucine degradation; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvG</i>	3.063866229	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvC</i>	4.371339203	valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
exp. phase		
<i>ybhC</i>	-3.005651583	pentose and glucuronate interconversions
<i>fhuF</i>	-2.013029339	ferric iron reductase
<i>fecD</i>	-1.925348313	iron-dicitrate ABC transporter subunit
<i>aceE</i>	-1.523183226	glycolysis; gluconeogenesis; citrate cycle (TCA cycle); pyruvate metabolism; lipoic acid metabolism
<i>ycdB</i>	-1.472910514	iron uptake system component
<i>aceF</i>	-1.398523034	glycolysis; gluconeogenesis; citrate cycle (TCA cycle); pyruvate metabolism; lipoic acid metabolism
<i>ycdO</i>	-1.354592654	iron uptake system component
<i>yqjH</i>	-1.302729542	ferric-chelate reductase
<i>efeU</i>	-1.289042204	ferrous iron permease
<i>fecA</i>	-1.188669015	ferric citrate outer membrane transporter
<i>exbB</i>	-1.172171856	enterochelin uptake protein
<i>sucB</i>	1.003798232	citrate cycle (TCA cycle); lysine degradation; tryptophan metabolism; lipoic acid metabolism
<i>dhaM</i>	1.010654719	glycerolipid metabolism
<i>gcvP</i>	1.084269904	glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; lipoic acid metabolism
<i>ilvC</i>	1.225445888	valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>glpK</i>	1.237027056	glycerolipid metabolism
ROK family protein	1.2491515	amino sugar and nucleotide sugar metabolism

(Continued)

TABLE 1 Continued

Gene name	log2-fold change	Pathway/function ^{82–84}
exp. phase		
<i>dsdA</i>	1.265931194	glycine, serine and threonine metabolism; D-Amino acid metabolism
<i>sucC</i>	1.271455252	citrate cycle (TCA cycle); propanoate metabolism; C5-Branched dibasic acid metabolism
<i>putP</i>	1.326926235	sodium/proline symporter
<i>gltI</i>	1.330564322	glutamate/aspartate transport system
<i>nadA</i>	1.36410938	nicotinate and nicotinamide metabolism
<i>glpT</i>	1.367466661	glycerol-3-phosphate transporter
<i>sucD</i>	1.382561251	citrate cycle (TCA cycle); propanoate metabolism; C5-Branched dibasic acid metabolism
<i>ygaW</i>	1.400831967	L-Alanine exporter
<i>glpQ</i>	1.436168876	glycerophospholipid metabolism
<i>glpD</i>	1.473785806	glycerophospholipid metabolism
<i>nanM</i>	1.493614699	N-acetylneuraminate epimerase
<i>gcvH</i>	1.5078413	glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; lipoic acid metabolism
<i>ilvD</i>	1.775787503	valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvA</i>	1.784324762	glycine, serine and threonine metabolism; valine, leucine and isoleucine biosynthesis
<i>dadX</i>	1.812119174	D-Amino acid metabolism
<i>srlD</i>	1.819297687	fructose and mannose metabolism
<i>putA</i>	1.857930281	alanine, aspartate and glutamate metabolism; arginine and proline metabolism
<i>nanK</i>	2.039366083	amino sugar and nucleotide sugar metabolism
<i>lldD</i>	2.043637543	pyruvate metabolism
<i>nanE</i>	2.076864669	amino sugar and nucleotide sugar metabolism
<i>ilvE</i>	2.167676205	cysteine and methionine metabolism; valine, leucine and isoleucine degradation; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvB</i>	2.647462771	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>yhcH</i>	2.806747795	N-acetylneuraminate anomerase
<i>ilvG</i>	3.332537625	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvN</i>	3.406210862	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
stat. phase		
<i>glcE</i>	-5.809515048	glyoxylate and dicarboxylate metabolism
<i>glcD</i>	-5.508308467	glyoxylate and dicarboxylate metabolism
<i>glcA</i>	-3.84055795	glycolate permease
<i>glcG</i>	-3.437418583	glycolate utilization
<i>glcB</i>	-3.040428269	pyruvate metabolism; glyoxylate and dicarboxylate metabolism
<i>alsA</i>	-2.970438825	D-allose transport ATP-binding protein
<i>glcF</i>	-2.686837435	glyoxylate and dicarboxylate metabolism
<i>garP</i>	-2.178038147	predicted (D)-galactarate transporter

(Continued)

TABLE 1 Continued

Gene name	log2-fold change	Pathway/function ^{82–84}
stat. phase		
<i>thrC</i>	-2.009914989	glycine, serine and threonine metabolism; vitamin B6 metabolism
<i>thrB</i>	-1.883274845	glycine, serine and threonine metabolism
<i>thrA</i>	-1.856342407	glycine, serine and threonine metabolism; cysteine and methionine metabolism; lysine biosynthesis; monobactam biosynthesis
<i>tdcG</i>	-1.747142565	glycine, serine and threonine metabolism; cysteine and methionine metabolism
<i>agaZ</i>	-1.742650224	galactose metabolism
<i>alsB</i>	-1.728052629	D-allose transporter subunit
<i>galS</i>	-1.637606696	transcriptional repressor and galactose ultrainduction factor
<i>garD</i>	-1.620616567	ascorbate and aldarate metabolism
<i>srlA</i>	-1.61111755	fructose and mannose metabolism
<i>ybhC</i>	-1.567162144	pentose and glucuronate interconversions
<i>mglB</i>	-1.542837699	D-galactose-binding periplasmic protein precursor
<i>mglA</i>	-1.531637382	ABC-type D-galactose transporter
<i>asnA</i>	-1.500124244	alanine, aspartate and glutamate metabolism; cyanoamino acid metabolism
<i>ssuB</i>	-1.413738896	sulfur metabolism
<i>srlD</i>	-1.38914946	fructose and mannose metabolism
<i>galE</i>	-1.350506556	galactose metabolism; amino sugar and nucleotide sugar metabolism; O-Antigen nucleotide sugar biosynthesis
<i>mglC</i>	-1.329707605	galactoside transport system permease protein
<i>melA</i>	-1.18645874	galactose metabolism; glycerolipid metabolism; sphingolipid metabolism
<i>asnB</i>	-1.065620685	alanine, aspartate and glutamate metabolism
<i>cdaR</i>	-1.061008612	carbohydrate diacid regulator
<i>lacZ</i>	-1.015924833	galactose metabolism; sphingolipid metabolism; other glycan degradation
<i>yahN</i>	-1.003789478	amino acid exporter
<i>adhP</i>	1.015337508	glycolysis; gluconeogenesis; pyruvate metabolism; fatty acid degradation; tyrosine metabolism; chloroalkane and chloroalkene degradation; naphthalene degradation
<i>guaB</i>	1.019960645	purine metabolism
<i>ilvA</i>	1.074184986	glycine, serine and threonine metabolism; valine, leucine and isoleucine biosynthesis
<i>aceK</i>	1.192060758	isocitrate dehydrogenase kinase/phosphatase
<i>ilvB</i>	1.778668589	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>mgtS</i>	1.992512123	Mg ²⁺ -import associated protein
<i>ilvG</i>	2.430932647	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>ilvN</i>	2.670495316	butanoate metabolism; C5-Branched dibasic acid metabolism; valine, leucine and isoleucine biosynthesis; pantothenate and CoA biosynthesis
<i>mgtA</i>	2.724809679	Mg ²⁺ -importing ATPase

metabolism. The *yghJ* (*ssIE*) gene, for example, codes for a metalloprotease contributing to mucin degradation in the intestinal tract or the bladder (Nesta et al., 2014). The most strongly downregulated genes were linked to glycolate utilization

(*glcDEFGBA*). Additionally, genes that are involved in D-allose transport (*alsAB*), galactarate metabolism (*garPD* and *cdaR*), galactose metabolism (*mglABC*, *lacZ*, *melA*, *agaZ* and *galES*), sorbitol utilization (*srlAD*), D-serine metabolism (*tdcG*) and

TABLE 2 Differentially expressed genes in *E. coli* 83972 *attB::lsr* relative to *E. coli* 83972 associated with motility, biofilm formation and adhesion.

Gene name	log2-fold change	Pathway/function ^{82–84}
lag phase		
<i>fliN</i>	-2.453087846	bacterial chemotaxis; flagellar assembly
<i>flgC</i>	-2.370643137	flagellar assembly
<i>fliG</i>	-2.154193018	bacterial chemotaxis; flagellar assembly
<i>fliA</i>	-1.967650727	biofilm formation; flagellar assembly
<i>fliP</i>	-1.934071506	flagellar assembly
<i>flgG</i>	-1.89377638	flagellar assembly
<i>fliE</i>	-1.817157817	flagellar assembly
<i>fliO</i>	-1.802383017	flagellar assembly
<i>flgN</i>	-1.76655253	flagellar assembly
<i>flgH</i>	-1.748868638	flagellar assembly
<i>flgB</i>	-1.704583646	flagellar assembly
<i>fliM</i>	-1.650792441	bacterial chemotaxis; flagellar assembly
<i>fliL</i>	-1.611497848	flagellar assembly
<i>fliH</i>	-1.518230416	flagellar assembly
<i>flgA</i>	-1.464314687	flagellar assembly
<i>flgD</i>	-1.426698341	flagellar assembly
<i>flgI</i>	-1.32985639	flagellar assembly
<i>fliZ</i>	-1.324136977	biofilm formation; flagellar assembly
<i>fliI</i>	-1.274724031	flagellar assembly
<i>fliA</i>	-1.24886028	flagellar assembly
<i>flgE</i>	-1.199949551	flagellar assembly
<i>fliF</i>	-1.178707702	flagellar assembly
exp. phase		
<i>bssS</i>	1.02819806	biofilm regulator; TA system
stat. phase		
<i>sfaX</i>	1.107129346	transcriptional regulator; S-fimbrial assembly
C terminal part of outer membrane F1C fimbrial usher protein SfaF	1.280095448	S-fimbrial assembly
<i>sfaS</i>	1.317340212	secretion system; S-fimbrial assembly
<i>sfaG</i>	1.364663339	adhesion; S-fimbrial assembly

(Continued)

TABLE 2 Continued

Gene name	log2-fold change	Pathway/function ^{82–84}
stat. phase		
<i>sfaY</i>	1.429358699	transcriptional regulator; S-fimbrial assembly
<i>sfaH</i>	1.475091904	secretion system; S-fimbrial assembly

sulfur utilization (*ssuB*) were significantly downregulated, too. Furthermore, genes associated with L-threonine (*thrABC*) and asparagine (*asnAB*) synthesis, as well as three genes that code for components of a putative oxidoreductase (*ydhYVX*) were also downregulated.

3.3 Reintroduction of the *lsr* locus reduces competitiveness *in vitro*

As the majority of differentially regulated genes affect the bacterial metabolism, we hypothesized that *E. coli* 83972 *attB::lsr* might have a growth defect when compared to the wild type strain 83972. Therefore, we analyzed and compared the growth behavior of both strains under aerobic and anaerobic conditions in LB (Figure 4) and pooled human urine (Supplementary Figure S3). In all conditions tested, *E. coli* 83972 *attB::lsr* had a significantly longer lag phase (Figures 4B, E; Supplementary Figures S3B, E) and a significantly higher doubling time than parental strain 83972 (Figures 4C, F; Supplementary Figures S3C, F). Next, we performed a growth competition experiment with *E. coli* 83972 and *E. coli* 83972 *attB::lsr* to test this phenotype for its biological relevance. To be able to differentiate between both strains by fluorescence microscopy, we introduced the plasmid pLS1 (*cfp* under the control of the stationary phase-dependent *dps* promoter) into *E. coli* 83972 *attB::lsr* and the plasmid pLS2 (identical to pLS1 on the nucleotide level, but *cfp* is replaced by *yfp*) into *E. coli* 83972. First, we mimicked bottleneck situations for which we mixed these two strains in a 1:1 ratio and grew the competing strains for 3 h, followed by a subsequent dilution into fresh medium. This cycle was repeated three times. After 3 h of growth, the proportion of *E. coli* 83972 (pLS2) increased from 52 ± 3.7% to 72 ± 13.3% while further increasing to 97 ± 0.8% after three transfers (Figure 5A). After 3 h of growth in pooled human urine, the proportion of *E. coli* 83972 (pLS2) rose from 49 ± 6.1% to 61 ± 2.1% while making up 79 ± 1.1% after three serial passages (Supplementary Figure S4A). We also tested for the long-term competitiveness of both strains. We observed that the proportion of *E. coli* 83972 (pLS2) significantly rose from 51 ± 1.7% to 75 ± 0.3% in LB after 24 h of direct competition (Figure 5B). Within the following 48 h, the proportion of *E. coli* 83972 (pLS2) remained unchanged. In pooled human urine, we saw that the proportion of the wild type strain

TABLE 3 Differentially expressed genes in *E. coli* 83972 *attB::lsr* relative to *E. coli* 83972 associated with growth.

Gene name	log2-fold change	Pathway/function ^{82–84}
lag phase		
<i>trnK3</i>	-1.936591094	aminoacyl-tRNA biosynthesis
<i>trnK4</i>	-1.904947039	aminoacyl-tRNA biosynthesis
<i>trnV3</i>	-1.772558046	aminoacyl-tRNA biosynthesis
<i>trnV5</i>	-1.713113393	aminoacyl-tRNA biosynthesis
<i>trnV4</i>	-1.708725917	aminoacyl-tRNA biosynthesis
<i>trnK1</i>	-1.324258994	aminoacyl-tRNA biosynthesis
<i>trnK2</i>	-1.217243916	aminoacyl-tRNA biosynthesis
<i>trnK5</i>	-1.150592884	aminoacyl-tRNA biosynthesis
<i>nrdF</i>	1.0340548	deoxyribonucleotide biosynthesis
<i>nrdH</i>	1.139857909	deoxyribonucleotide biosynthesis
<i>nrdE</i>	1.269219097	deoxyribonucleotide biosynthesis
<i>nrdI</i>	1.30323686	deoxyribonucleotide biosynthesis
exp. phase		
<i>trnK3</i>	-1.676797599	aminoacyl-tRNA biosynthesis
<i>trnK4</i>	-1.60586492	aminoacyl-tRNA biosynthesis
<i>trnV4</i>	-1.238175595	aminoacyl-tRNA biosynthesis
<i>trnV5</i>	-1.23717919	aminoacyl-tRNA biosynthesis
<i>trnV3</i>	-1.237008793	aminoacyl-tRNA biosynthesis
<i>pyrE</i>	-1.194313829	pyrimidine <i>de novo</i> biosynthesis
<i>cydB</i>	-1.006975401	cytochrome d ubiquinol oxidase subunit
stat. phase		
<i>rpsS</i>	-1.193234994	component of 30S ribosomal subunit
<i>dbpA</i>	-1.087482054	ribosome biogenesis
<i>ybjG</i>	1.166583523	peptidoglycan biosynthesis; teichoic acid biosynthesis

significantly rose from $48 \pm 2.8\%$ to $67 \pm 2.7\%$ after 72 h of competition (Supplementary Figure S4B).

3.4 Reintroduction of *lsr* locus or deletion of *ybhC* leads to a lower resistance against oxidative stress

To test for resistance to oxidative stress, we added different concentrations of H₂O₂ to low bacterial numbers of the *E. coli* strains 83972, 83972 $\Delta ybhC$ and 83972 *attB::lsr* and analyzed their growth behavior. We observed that the length of the lag phase was significantly longer for *E. coli* 83972 after the addition of 160 μ M H₂O₂ as compared to the water control, while the addition of 40 μ M or 100 μ M did not lead to a significantly longer lag phase. In the case

TABLE 4 Differentially expressed genes in *E. coli* 83972 *attB::lsr* relative to *E. coli* 83972 associated with stress response.

gene name	log2-fold change	pathway/function ^{82–84}
lag phase		
<i>cpxP</i>	-1.012637356	chaperones and folding catalysts; resistance to extracytoplasmic stress
exp. phase		
<i>sodA</i>	-1.132854292	superoxide dismutase
<i>ycjX</i>	1.056824975	induced under nitrogen starvation
<i>inaA</i>	1.254017352	pH-inducible protein involved in stress response
<i>ibpB</i>	2.077026895	heat shock protein
stat. phase		
<i>ydhY</i>	-1.674500305	predicted ferredoxin-like protein
<i>ydhX</i>	-1.481012743	predicted ferredoxin-like protein
<i>ydhV</i>	-1.131341197	aldehyde ferredoxin oxidoreductase
<i>marB</i>	1.133272934	multiple antibiotic resistance protein
<i>ymdF</i>	1.250735552	stress-induced protein
<i>ycfR</i>	1.262325635	stress-response protein

of the *E. coli* strains 83972 $\Delta ybhC$ and 83972 *attB::lsr*, on the other hand, already the addition of 100 μ M resulted in significantly longer lag phases as compared to the corresponding water controls (Figure 6). Once the strains entered exponential growth, however, the doubling times were not affected by the initial H₂O₂ stress (Supplementary Figure S5).

3.5 Complementation of *E. coli* strain 83972 with the *lsr* operon results in impaired colonization capacity in the digestive tract of *Galleria mellonella* larvae

Uropathogenic *E. coli* normally have their reservoir in the densely populated intestinal tract, and urinary tract infections usually occur through smear infection with bacteria originating from the intestinal tract. To test whether the presence of a functional *lsr* operon affects the ability of *E. coli* strain 83972, as a degenerate uropathogen, to efficiently colonize a niche that is densely populated by different types of bacteria such as the intestinal tract, we fed *G. mellonella* larvae with a mixture of *E. coli* strain 83972 and its *lsr*-complemented variant in a 1:1 ratio. Quantification after 24 h incubation showed that the *lsr*-positive *E. coli* 83972 variant was found in significantly lower numbers in the digestive tract of the *G. mellonella* larvae than the wild type strain. After 24 hours, the *lsr*-negative wild type *E. coli* 83972 outcompeted its *lsr*-positive complemented counterpart in the digestive tract of *G. mellonella* larvae (Figure 7).

TABLE 5 Remaining differentially expressed genes in *E. coli* 83972 *attB*::*lsr* relative to *E. coli* 83972.

Gene name	log2-fold change	Pathway/function ^{82–84}
lag phase		
putative methyltransferase	-2.044036679	–
antirestriction protein	-1.674016348	–
<i>yabN</i>	-1.441756062	transcription factor
<i>yhfG</i>	-1.409124762	TA System
<i>ycjF</i>	-1.10548474	predicted membrane protein associated with virulence in a murine model
<i>ymgD</i>	-1.024673712	possibly TA system
<i>lsrR</i>	6.259241476	transcriptional repressor; <i>lsr</i> locus introduction
<i>lsrK</i>	6.922763675	AI-2 kinase; <i>lsr</i> locus introduction
<i>aph</i>	11.20318319	kanamycin resistance gene; <i>lsr</i> locus introduction
<i>cat</i>	11.59026441	chloramphenicol resistance gene; <i>lsr</i> locus introduction
exp. phase		
<i>ymgD</i>	-2.028703146	possibly TA system
hypothetical protein 10	-1.823290623	–
hypothetical protein 6	-1.662041435	–
hypothetical protein 8	-1.33921833	–
<i>yiaD</i>	-1.267749645	putative outer membrane protein
<i>pmrR</i>	-1.155320974	small regulatory membrane protein
outer membrane porin protein LC precursor	1.100938115	transporter; pores iron channel
<i>fxsA</i>	1.275237522	cytoplasmic membrane protein
<i>ompF</i>	1.328798394	outer membrane protein
<i>lsrR</i>	8.021393949	transcriptional repressor; <i>lsr</i> locus introduction
<i>lsrK</i>	8.277667483	AI-2 kinase; <i>lsr</i> locus introduction
<i>aph</i>	11.13917456	kanamycin resistance gene; <i>lsr</i> locus introduction
<i>cat</i>	13.30014141	chloramphenicol resistance gene; <i>lsr</i> locus introduction
stat. phase		
<i>yghJ</i>	-2.038713336	putative lipoprotein
<i>yjaB</i>	-1.080319092	peptidyl-lysine <i>N</i> -acetyltransferase
hypothetical protein 12	-1.031550868	–
<i>yrbL</i>	1.010712982	–

(Continued)

TABLE 5 Continued

Gene name	log2-fold change	Pathway/function ^{82–84}
stat. phase		
antirestriction Ral family protein	1.033440867	–
putative colicin immunity protein	1.061726625	–
hypothetical protein 7	1.370170227	–
VapA/VapB family virulence associated protein	1.892144739	–
<i>lsrG</i>	6.851703901	isomerase; <i>lsr</i> locus introduction
<i>lsrC</i>	8.029960122	part of AI-2 transporter; <i>lsr</i> locus introduction
<i>lsrB</i>	8.088569874	AI-2 receptor; <i>lsr</i> locus introduction
<i>lsrF</i>	8.122185059	thiolase; <i>lsr</i> locus introduction
<i>lsrD</i>	8.795794664	part of AI-2 transporter; <i>lsr</i> locus introduction
<i>lsrA</i>	9.402359596	part of AI-2 transporter; <i>lsr</i> locus introduction
<i>lsrK</i>	9.814535263	AI-2 kinase; <i>lsr</i> locus introduction
<i>lsrR</i>	10.53084923	transcriptional repressor; <i>lsr</i> locus introduction
<i>aph</i>	11.52317813	kanamycin resistance gene; <i>lsr</i> locus introduction
<i>cat</i>	13.6273497	chloramphenicol resistance gene; <i>lsr</i> locus introduction

3.6 *E. coli* of the phylogroup B2 are predominantly *lsr*-negative

To obtain an overview of the abundance of the *lsr* locus in the *E. coli* population, we screened all *E. coli* genomes that were available from the NCBI database concerning their phylogroup and the presence of the full-length *lsr* locus. In total, we analyzed 32,404 genomes of isolates from the phylogroups A, B1, B2, C, D, E, F, and G. Genomes from isolates of phylogroup B1 accounted for the largest share (29.1%), followed by phylogroup A (28.2%) and phylogroup B2 (18.6%) (Supplementary Table S2). We screened these genomes for the presence of the full-length *lsr* locus (reference DNA sequence from the *E. coli* K-12 strain MG1655) in all genomes. The search resulted in 22,259 complete, 1,861 incomplete and 2,400 multiple matches. In 5,884 genomes, we detected no match or a blastn hit length that was shorter than 100 bp (Supplementary Table S3). We found that > 81% of *E. coli* strains belonging to the phylogroups A1, B1, C, D, E, F, or G carry a full-length *lsr* locus. On the contrary, in > 95% of *E. coli* isolates belonging to the phylogroup B2, a homologous region smaller than

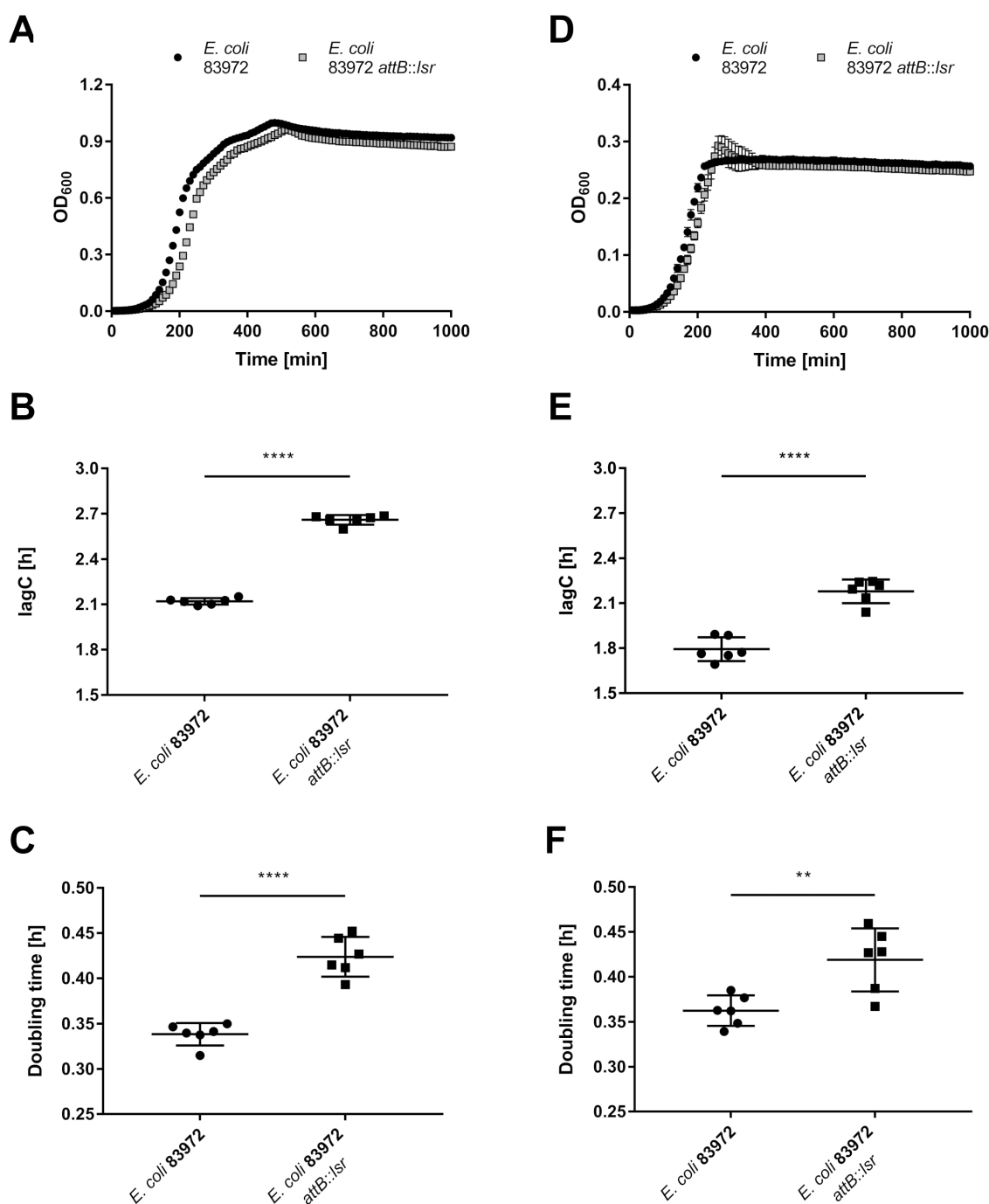


FIGURE 4

Restoration of the *lsr* determinant impairs growth of *E. coli* 83972. Growth analysis of *E. coli* strains 83972 and 83972 *attB::lsr* was done in LB under (A–C) aerobic and (D–F) anaerobic conditions. (A, D) Growth curves over a time span of 1000 min with optical density (OD₆₀₀) measurements every 10 min of *E. coli* 83972 (black circles) and *E. coli* 83972 *attB::lsr* (grey squares). (B, E) Time until the cultures reached the exponential growth phase (lagC). (C, F) Doubling time during the exponential growth phase. Depicted are three biological replicates performed in duplicates each. The starting OD₆₀₀ was 0.01 for both strains. Growth curve analysis was done using AMiGA (Midani et al., 2021). Statistical analysis was performed using an unpaired t-test; values < 0.05 were considered statistically significant (** p < 0.01, **** p < 0.0001).

100 bp was detected (Figure 8, Supplementary Figure S6A). We analyzed the prevalence of the *luxS* and *tam* genes in the *E. coli* population. The search for the *luxS* genes in the genome collection resulted in 32,301 complete, 22 incomplete, and 62 multiple matches. In 19 genomes, we found no match (Supplementary

Table S3). The search for the *tam* gene, which is located downstream of *lsrG* resulted in 30,364 complete, 1,679 incomplete, and 181 multiple matches. In 180 genomes, we found no match (Supplementary Table S3). Accordingly, more than 99% of the analyzed *E. coli* genomes include a full-length *luxS* gene

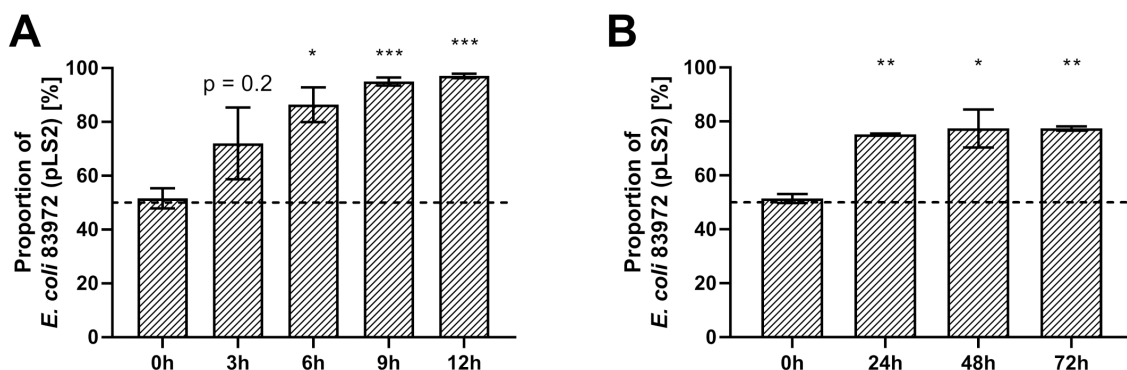


FIGURE 5

Restoration of the *lsr* determinant reduces competitiveness in serial passaging of *E. coli* 83972. Competition assays were done by mixing overnight cultures of strains 83972 (pLS2) and 83972 *attB::lsr* (pLS1) in a 1:1 ratio in LB. (A) The cultures were grown and subsequently diluted (1:200) into fresh medium every 3 h. Depicted are the mean values and standard deviations of the ratio analysis of ten microscopic pictures for four biological replicates at the indicated time points. (B) The cultures were grown over a total of 72 h. Depicted are the mean values and standard deviations of the ratio analysis of ten microscopic pictures for three biological replicates at the indicated time points. Statistical analysis was performed using RM one-way ANOVA (Geisser-Greenhouse correction) with Dunnett's multiple comparison test; values < 0.05 were considered statistically significant (* p<0.05, ** p<0.01, *** p<0.001).

(Supplementary Figure S6B), and > 88% of the analyzed *E. coli* genomes possess a full-length *tam* gene (Supplementary Figure S6C).

4 Discussion

AI-2-dependent QS was shown to play an essential role in APEC and MPEC strains for virulence, colonization, or resistance (Palaniyandi et al., 2013; Han et al., 2015; Xue et al., 2016; Zuo et al., 2019; Yu et al., 2020; Wang et al., 2021; Helmy et al., 2022). However,

except for knowledge about the role of QS-induced biofilm formation in catheter-associated UTIs (Laganenka and Sourjik, 2018; Henly et al., 2021; Lila et al., 2023), little is known about the function of AI-2-dependent QS in UTIs. *E. coli* 83972 is naturally *lsr*-negative but *luxS*-positive and causes asymptomatic bacteriuria (Medina and Castillo-Pino, 2019). We analyzed this strain's ability to produce, export, import, and phosphorylate AI-2. We found that *E. coli* 83972 produced AI-2, which could be sensed by another *E. coli* strain during co-culture (Figure 2B). The AI-2 production was LuxS-dependent, as judged by the inability to induce AI-2-dependent YFP expression in our reporter strain when *luxS* was deleted in *E. coli* 83972

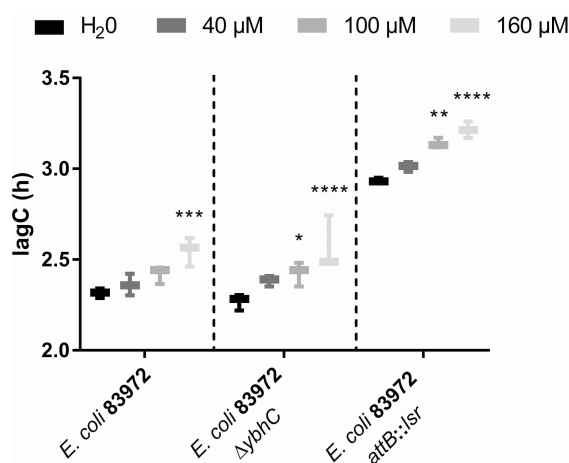
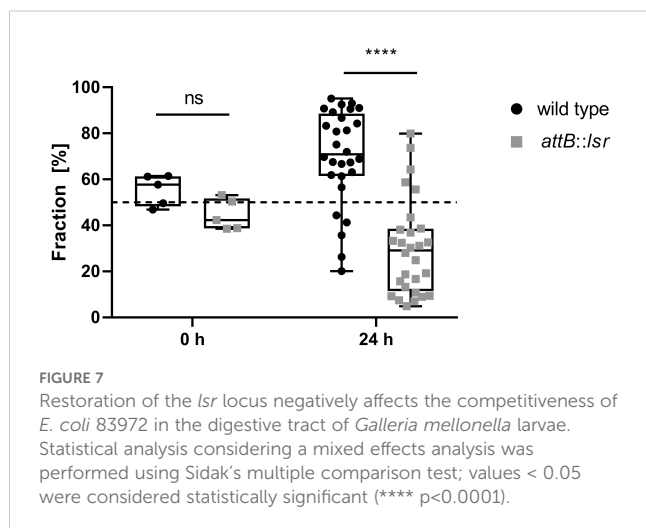
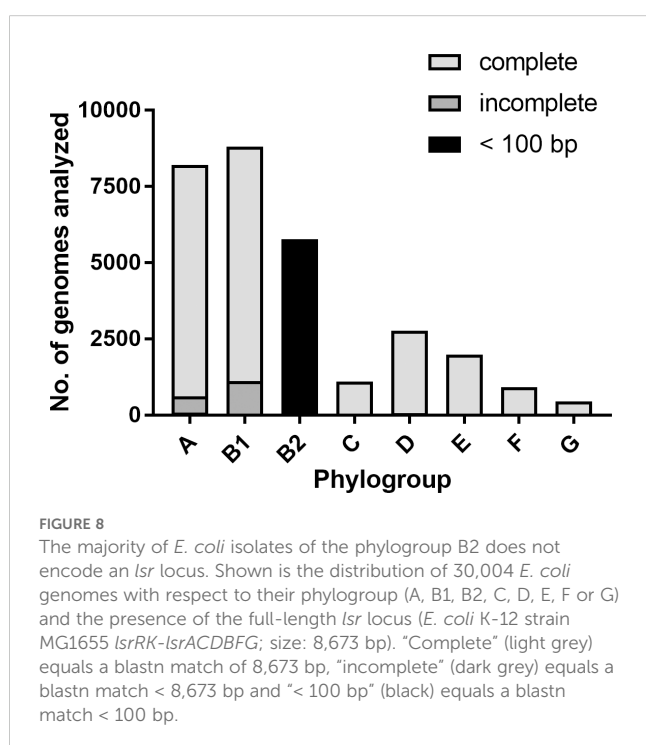


FIGURE 6

Introduction of the *lsr* locus or deletion of *ybhC* negatively affects oxidative stress resistance of *E. coli* 83972. Shown is the time until the bacteria reach the exponential growth phase (lagC) during growth of *E. coli* strains 83972, 83972 $\Delta ybhC$ and 83972 *attB::lsr* in LB. The starting OD₆₀₀ was 0.01 for each strain. Before growth analysis, the strains were challenged with a final H₂O₂ concentration of 40 μM, 100 μM or 160 μM. Water was added as a negative control. LagC was analyzed using AMiGA (Midani et al., 2021). The statistical analysis was performed using ordinary two-way ANOVA with Tukey's multiple comparison test. Only the effect of different H₂O₂ concentrations on the growth of the respective *E. coli* strains was compared (83972, 83972 $\Delta ybhC$ and 83972 *attB::lsr*; simple effect within rows); values < 0.05 were considered statistically significant (** p<0.01, *** p<0.001, **** p<0.0001).



(Figure 2B). However, we observed no AI-2-dependent YFP expression when *E. coli* 83972 $\Delta luxS$ (pMK2) was challenged with extracellular DPD (Figures 2D, E). In comparison, the addition of low concentrations of extracellular DPD to *E. coli* 83972 $\Delta luxS$ (pMK1) has led to a significantly higher YFP expression compared to the water control (Figure 2F). This result was expected since *E. coli* 83972 does not encode LsrK, which is so far the only described kinase that phosphorylates incorporated AI-2 (Pereira et al., 2012). Since AI-2 is initially taken up in an Lsr-independent way by a phosphoenolpyruvate phosphotransferase system, the lack of an active AI-2 kinase would not lead to an AI-2 sequestration inside the cell because unphosphorylated AI-2 is transported back into the extracellular space (Marques et al., 2011; Pereira et al., 2012; Trappetti et al., 2017). When pMK1, a plasmid encoding LsrK, was introduced into *E. coli* 83972, externally



added AI-2 was incorporated, phosphorylated, and sequestered inside the cell, while the introduction of pMK2, lacking *lsrK*, did not lead to AI-2 phosphorylation. These results let us conclude that *E. coli* 83972 produces AI-2, which can be sensed by other bacteria but cannot process AI-2 itself. This is not unusual, as other bacteria are also known to be unable to sense AI-2 and express *luxS* only because of its role in the activated methyl cycle (Rezzonico and Duffy, 2008). One example is the uropathogenic *Proteus mirabilis* strain BB2000, which does not possess a *luxP* or *lsrB* homolog but does express *luxS* (Rezzonico and Duffy, 2008). Furthermore, the deletion of *luxS* did not lead to altered colonization or pathogenicity of this strain during UTI in a mouse model (Schneider et al., 2002), suggesting that AI-2-dependent QS does not play a role during UTI in this strain. To reintroduce the ability to use AI-2 as a signaling molecule, we integrated the *lsr* locus into the chromosomal *attB* site of *E. coli* 83972 (Figure 2A). Indeed, when *E. coli* 83972 $\Delta luxS$ *attB::lsr* (pMK2) was challenged with extracellular DPD, we observed a significantly higher YFP expression as compared to the water control (Figures 2D, E). This result suggests that *lsrK* is functionally expressed in *E. coli* 83972 *attB::lsr*. Additionally, *E. coli* 83972 *attB::lsr* could export AI-2, which could be sensed by another *E. coli* strain in coculture (Figure 2C).

The observation that global gene expression of UPEC strains *in vivo* during human UTI (hUTI) or murine UTI (mUTI) was more similar to their *in vitro* gene expression in LB than to their *in vitro* gene expression in pooled human urine (Frick-Cheng et al., 2020) allows us to assess the importance of *lsr*-dependent gene expression for colonization of the urinary bladder using our own *in vitro* RNA-seq data in LB. Our transcriptome analysis revealed that the transcriptomes of the *E. coli* strains 83972 and 83972 *attB::lsr* were not drastically different during the three growth phases (Supplementary Figure S2). Only 60–72 genes were differentially expressed in *E. coli* strains 83972 and 83972 *attB::lsr* with a log₂-fold change of higher or lower 1 (Figure 3). By comparing the results of Frick-Cheng and colleagues (Frick-Cheng et al., 2020) to our data, we observed that some genes or operons that were differentially expressed *in vivo* during hUTI/mUTI relative to *in vitro* growth in LB, also seem to be affected by AI-2-dependent QS. For example, several genes from the flagellar machinery (*flgCFGLM* and *fliS*) were downregulated during mUTI/hUTI as compared to growth in LB (Frick-Cheng et al., 2020). More than 50% of all downregulated genes in *E. coli* 83972 *attB::lsr* in the lag phase were linked to the flagellar machinery (Table 2). The link between AI-2-dependent QS and flagellar gene expression was also seen in the EHEC strain 86-24, where the deletion of *luxS* led to a downregulation of flagellar genes (Sperandio et al., 2001), which is contradictory to our results where AI-2-dependent QS seems to repress flagellar gene expression. In UPEC strain CFT073, flagella expression was not crucial for bladder colonization efficiency, whereas the loss of flagellar genes was a disadvantage during bladder colonization (Lane et al., 2005). During UTI, the flagella induce a Toll-like receptor-dependent immune response and, thus, inflammation (Subashchandrabose and Mobley, 2015; Acharya et al., 2019). However, since the *flhDC* genes coding for the master regulator of the flagellar biosynthesis cascade (Subashchandrabose and Mobley, 2015), and the highly antigenic flagellin, encoded by *fliC*

(Acharya et al., 2019), were not differentially expressed in *E. coli* 83972 *attB::lsr* (Table 2, Supplementary Table S1), and since *E. coli* 83972 expresses only little flagella (Hancock et al., 2008), it appears unlikely that the further downregulation of already weakly expressed flagellar genes is negatively impacting this strain's bladder colonization ability.

One of the main characteristics of *E. coli* 83972 is its fast growth in urine and, thus, the outgrowth of competing UPEC strains in direct competition (Roos et al., 2006; Ipe et al., 2016). One factor that might contribute to this higher competitiveness is metabolic adaptation (Ipe et al., 2016). We found several genes associated with metabolism to be differentially expressed in all three growth phases in *E. coli* 83972 *attB::lsr* (Table 1). AI-2-dependent QS was already linked to sugar metabolism (Ha et al., 2018), catabolite repression (Wang et al., 2005) and carbon regulation (Mitra et al., 2016). The strong upregulation of genes involved in the valine, leucine, and isoleucine biosynthesis (*ilv* operon) was interesting since *ilvA* and *ilvC* were found to be essential factors during growth in urine (Vejborg et al., 2012), probably because the overall concentrations of the three amino acids are relatively low as compared to other amino acids in urine (Bouatra et al., 2013) (Table 1, Supplementary Table S1). In *E. coli* strain CFT073, valine overproduction is considered a metabolic adaptation during biofilm formation (Valle et al., 2008). We also found several genes related to biofilm formation and adhesion upregulated in the transition to the stationary phase upon restoration of the *lsr* operon (Supplementary Table S1, Table 2). Thus, we checked for biofilm formation according to the protocol of (Laganenka et al., 2016). In *E. coli* strains 83972 and 83972 *attB::lsr*, but observed no significant differences (Supplementary Figure S7). Thus, the observed *lsr*-dependent upregulation of the *ilv* genes had no marked effect on biofilm formation of *E. coli* strain 83972 *attB::lsr*.

We wondered whether the differential regulation of metabolic genes in *E. coli* 83972 *attB::lsr* might affect growth. Indeed, we observed a growth retardation of this strain compared to the *lsr*-negative wild type 83972, independent of growth medium or oxygen availability (Figure 4, Supplementary Figure S3). Consistent with the findings of Sperandio and colleagues made in EHEC strain 86-24 (Sperandio et al., 2001), we observed that the deletion of *luxS* resulted in a significantly longer lag phase of *E. coli* strain 83972 (Supplementary Figure S8). Interestingly, the restoration of the *lsr* determinant in *E. coli* 83972 significantly extended the lag phase as well as the doubling time of *E. coli* 83972 (Supplementary Figure S8). As we did not observe an additive effect of *luxS* deletion and *lsr* restoration on the length of the lag phase in *E. coli* 83972, and since *luxS* was not differentially expressed in *E. coli* 83972 *attB::lsr* in all three growth phases, the longer lag phase of the strains 83972 Δ *luxS* and 83972 Δ *luxS attB::lsr* as compared to the wild type might thus be due to the loss of LuxS as metabolic enzyme (Schauder et al., 2001; Vendeville et al., 2005; Zdzarski et al., 2008) and not due to AI-2 secretion, as *E. coli* 83972 and *E. coli* 83972 Δ *luxS* are not able to sense AI-2. Taken together, these findings showed that the presence of the full-length *lsr* locus alone was not sufficient to cause the observed slowdown in growth but that it most likely resulted from an AI-2-dependent deregulation of specific gene expression. Because we

performed the complementation as a single copy-insertion of the *lsr* determinant into a neutral site of the chromosome, overexpression effects and artificial phenotypes due to incompatibilities in gene regulation or metabolic stress caused by overexpression should be excluded. To validate our assumption, additional *E. coli* isolates will have to be tested for their *lsr*-dependent growth characteristics. In this context, fitness costs from the two *cat* and *aph* resistance cassettes used for complementation could be considered as well as non-QS-related functions of the *lsr* determinant.

In the intestinal tract, where the bacterial composition is diverse and the bacterial load is high, a negative effect of AI-2-dependent QS on growth rate does not necessarily lead to a reduction in fitness as AI-2-dependent QS significantly impacts microbiota composition, which in turn affects the fitness of individual bacterial species (Christiaen et al., 2014; Thompson et al., 2015; Deng et al., 2022). Nevertheless, our feeding experiments of *G. mellonella* larvae with equal ratios of *E. coli* 83972 wild type and the *lsr*-complemented strain impressively showed that the restoration of the *lsr* operon significantly reduced the colonization ability and competitiveness of *E. coli* 83972 *attB::lsr* in the digestive tract compared to the *lsr*-negative parent strain. We are fully aware that the human intestinal tract and that of *G. mellonella* larvae differ substantially, not only in their microbiome composition. We applied *G. mellonella* larvae as a 3R-compliant model to investigate the ability of *E. coli* 83972 to assert itself in a niche densely populated by a complex microbiota. We view this experiment as a proxy for intestinal colonization and understand that a more relevant colonization model for the human digestive tract must be used in further studies to clarify the impact of AI-2-dependent QS on the intestinal colonization capacity of phylogroup B2 strains. It has recently been described in a mouse model that AI-2 production increases chemotaxis and thus the metabolic trait-dependent fitness of *E. coli* strain Z1331 in the intestine. AI-2-dependent chemotaxis can also promote the coexistence of different *E. coli* strains in the intestine through niche segregation (Laganenka et al., 2023). Related to hardly flagellated *E. coli* strain 83972, we interpret our results as indicating that the importance of AI-2 in bacterial fitness in the digestive tract can be strain-dependent and influenced by individual metabolic and phenotypic characteristics. In the human bladder, however, where bacterial numbers are typically not as high as in the intestinal tract (Perez-Carrasco et al., 2021), regulating growth by monitoring cell density may be a disadvantage for urine isolates, because of rapid changes of the population size in the bladder. The urine void drastically reduces the overall bacterial count and volume of the growth compartment, urging non-voided bacteria to regrow fast to maintain bladder colonization, especially when competing with a second bacterial species (Ipe et al., 2016). QS is known to slow down growth and metabolism as soon as a high cell density is reached (Sperandio et al., 2001; An et al., 2014). When the bacteria encounter a rapid reduction in cell density, this switch leads to a subsequent adaptation of gene expression in quorum sensing strains (Pereira et al., 2013), which likely differs from that in bacteria that cannot sense the bacterial cell density. In our co-cultivation experiment,

where the *lsr*-positive strain 83972 *attB::lsr* (pLS1) and the *lsr*-negative strain 83972 (pLS2) were in direct competition, the *lsr*-positive variant capable of QS was outcompeted by the isogenic *lsr*-negative wild type, which is unable to sense bacterial cell density but displays a shorter lag phase and doubling time (Figure 4). The observed slowdown in growth and prolonged doubling time of the *lsr*-positive variant could underlie the displacement of this mutant by the QS-insensitive wild type due to QS-dependent adaptations in gene expression. Especially in the case of frequent changes in population density, here mimicked by serial passage after 3 h of growth (Figure 5A), compared to the long-term competition experiment (Figure 5B), the displacement by the *lsr*-negative strain is particularly evident. This property benefits *E. coli* 83972 in bacterial interference in the urinary bladder because it can compensate for bottlenecks due to rapid reductions in population size as a result of repeated voiding. In summary, our results suggest that the differential expression of metabolic genes by AI-2-dependent QS may result in different growth characteristics and reduced competitiveness of *E. coli* 83972 *attB::lsr*. The direct evidence, however, is still missing, and the precise regulatory mechanisms remain to be elucidated.

The *nrdHIEF* operon was upregulated during mUTI/hUTI as compared to growth in LB (Frick-Cheng et al., 2020), and we found *nrdHIEF* upregulated in *E. coli* 83972 *attB::lsr* as compared to *E. coli* 83972 in the lag phase (Table 3). Though the operon encodes a ribonucleotide reductase that is required for dNTP synthesis and is thus associated with DNA synthesis, *nrdHIEF* expression was found to be stimulated upon oxidative stress, particularly in mutants that miss major antioxidant defenses (Monje-Casas et al., 2001). It is thought that *E. coli* overexpresses several reductases and electron donors, including *nrdHIEF*, to cope with oxidative stress (Monje-Casas et al., 2001; Abdelwahed et al., 2022). Interestingly, the gene *ybhC*, which leads to a significantly lower minimal inhibitory concentration against H₂O₂ when deleted in the *E. coli* strain BW25113 (Chen et al., 2021), was the most strongly downregulated gene during the lag and exponential growth phase and was also strongly downregulated during the transition to the stationary phase in *E. coli* 83972 *attB::lsr* (Supplementary Table S1). We wondered whether this strong downregulation might contribute to a higher sensitivity to H₂O₂. Therefore, we challenged *E. coli* 83972, its isogenic *ybhC* deletion mutant 83972 $\Delta ybhC$, and *E. coli* 83972 *attB::lsr* with H₂O₂ concentrations that can be physiologically measured in human urine (Varma and Devamanoharan, 1990). Indeed, we observed that *E. coli* 83972 was more resistant to H₂O₂ than the strains 83972 $\Delta ybhC$ and 83972 *attB::lsr* (Figure 6). We observed that the *ybhC* deletion did not affect the length of the lag phase as compared to *E. coli* 83972 (unpaired t-test; $p = 0.19$), whereas *E. coli* 83972 *attB::lsr* had a significantly longer lag phase than both, *E. coli* 83972 and *E. coli* 83972 $\Delta ybhC$ (Figure 6). Thus, the overall longer lag phase of the *lsr*-positive variant of strain 83972 does not explain its higher sensitivity to H₂O₂, while the strong downregulation of *ybhC* in this strain might be one factor responsible for this phenotype. Notably, the increased H₂O₂ sensitivity of *E. coli* 83972 *attB::lsr* is in agreement with a previous report of increased H₂O₂ sensitivity in the MPEC strain

DCM5 expressing a functional LsrR (Wang et al., 2021). However, in this MPEC strain, the higher sensitivity was explained by LsrR-mediated repression of H₂O₂ scavenging enzyme-encoding genes, including *ahpCF*. As opposed to that, we found that *ahpCF* was slightly upregulated in strain 83972 *attB::lsr* in the lag phase (Supplementary Table S1). Therefore, the decreased expression of *ahpCF* is unlikely to explain the *lsr*-dependent sensitivity of *E. coli* 83972 to H₂O₂. *E. coli* 83972 has an increased level of endogenous reactive oxygen species while growing in urine compared to other ABU and UPEC strains, but correspondingly, it also has a more active antioxidant defense system (Aubron et al., 2012). We found several differentially expressed genes in *E. coli* 83972 *attB::lsr* that have been associated with oxidative stress in other studies (Supplementary Table S4) or other stress-related responses (Table 4). Thus, the overall H₂O₂ detoxification or stress response seems to be imbalanced in the presence of functional AI-2-dependent QS in *E. coli* 83972 *attB::lsr*, resulting in a higher sensitivity against oxidative stress. During ABU, however, increased sensitivity to H₂O₂ is a disadvantage since the infiltration of activated neutrophils into the bladder represents one host defense mechanism to kill bacteria with reactive oxygen species during UTI (Demirel et al., 2020). Especially in a competition situation in the bladder, a significantly prolonged lag phase and doubling time of *E. coli* 83972 *attB::lsr* in addition to the increased H₂O₂ sensitivity could constitute a clear colonization disadvantage relative to the *lsr*-negative wild type strain (Supplementary Figure S5).

AI-2-dependent QS in ExPEC has pleiotropic effects since it contributes to virulence, colonization, and antibiotic resistance but also H₂O₂ sensitivity, metabolism, and growth defects, as can also be seen in our study (Xue et al., 2009; Kathayat et al., 2021; Wang et al., 2021). Epidemiological studies have shown that ExPEC frequently belong to the phylogroup B2 (Picard et al., 1999; Micenková et al., 2016). However, in the collection of *E. coli* genomes we examined, genomes of phylogroup A and B1 isolates were predominant (Supplementary Table S2). Intriguingly, it was already suggested that *E. coli* strains of the phylogroup B2 might have lost the *lsr* locus during evolution (Quan and Bentley, 2012; Brito et al., 2013). However, all B2 strains analyzed by Brito and colleagues encoded a functional LuxS synthase and thus should be capable of producing AI-2 (Bruto et al., 2013). Following Brito and colleagues, we found that the vast majority of *E. coli* belonging to the phylogroup B2 lack a full-length *lsr* locus (Figure 8, Supplementary Figure S6A), while the presence of *luxS* seems to be remarkably conserved (Supplementary Figure S6B). The high prevalence of *luxS* in *E. coli* and also over a long time in bacteria (Santiago-Rodriguez et al., 2014) underlines its crucial role as a metabolic enzyme in the activated methyl cycle (Vendeville et al., 2005). The *tam* gene, which is located downstream of the *lsrACDBFG* operon, was also widely distributed (> 88%) in the different phylogroups (Supplementary Figure S6C). Since *E. coli* 83972 encodes Tam and its role in AI-2-dependent QS remains unclear, we omitted *tam* from the complemented *lsr* locus. Indeed, we observed that *tam* was upregulated during the transition to the stationary phase (data not shown) but was not differentially

expressed in *E. coli* 83972 *attB::lsr* as compared to *E. coli* 83972. Evolutionary studies suggest that the *lsr* locus was passed on via horizontal gene transfer (Rezzonico et al., 2012), and lateral gene transfer events were also described (Pereira et al., 2009). Thus, it is interesting that the operon was not reintroduced into the phylogroup B2 (Manges and Johnson, 2015). These observations and our data suggest that the loss of AI-2-dependent QS could be an evolutionary advantage for *E. coli* 83972, which efficiently colonizes the urinary bladder as an extraintestinal body niche. Whether the absence of the *lsr* determinant in almost all phylogroup B2 isolates investigated so far may be correlated, at least in part, with generally increased fitness properties in extraintestinal niches or superior colonization properties in densely populated niches such as the intestinal tract will require more detailed future analyses of the effect of *lsr* complementation in a diverse collection of phylogroup B2 strains.

5 Conclusion

It is imperative that we explore non-antibiotic solutions to prevent UTI, with bacterial interference being a promising avenue for treating symptomatic episodes of uncomplicated cystitis. Our study provides new information on gene regulation in *E. coli* 83972. The more we understand which bacterial characteristics improve fitness and colonization properties in the urinary bladder, the better we could rationally improve the engineering of *E. coli* 83972 or predict outcomes in therapeutic colonization. The impact of AI-2-mediated QS for pathogenicity or fitness of ExPEC is not uniform and is probably dependent on the individual strain background. Whereas interference with AI-2-dependent QS resulted in downregulation of ExPEC virulence traits and attenuation in some isolates, increased fitness due to enhanced resistance to oxidative stress was observed in others. It is tempting to speculate that the absence of the *lsr* operon in the majority of phylogroup B2 isolates may potentially suggest that the loss of AI-2-dependent QS could be associated with a fitness advantage in extraintestinal niches or their reservoir, i.e., the intestinal tract. However, this must be further analyzed in detail in the future. In this study, we investigated the impact of AI-2-mediated QS on fitness traits of asymptomatic bacteriuria *E. coli* isolate 83972, a strain that has already been used for therapeutic bladder colonization, thereby interfering with bladder infections caused by uropathogens. We found that the reintroduction of AI-2-dependent QS in *E. coli* 83972, which is optimally adapted to the growth conditions in the urinary bladder, has led to a phenotype that is disadvantageous for efficient and long-term bladder colonization, especially in a competitive situation. Restoration of the *lsr* determinant in *E. coli* 83972 resulted in growth retardation, loss of competitiveness and increased susceptibility to oxidative stress, all characteristics relevant to this strain's colonization ability in the bladder. Thus, our findings indicate that the absence of AI-2-dependent QS in *E. coli* 83972 may be an advantage during the colonization of the urinary bladder. Further studies on the benefits of the lack of the *lsr* determinant in a broad spectrum of ExPEC isolates with different phylogenetic

backgrounds and genome content are needed to gain deeper insights into the general importance of AI-2-dependent QS for *E. coli* fitness and pathogenicity in extraintestinal niches and or the colonization of the intestinal tract.

Data availability statement

The datasets presented in this study are publicly accessible at NCBI GEO (SRA accession number GSE300954). This data can be found here: (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE300954>).

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

MK: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. KM: Data curation, Investigation, Methodology, Writing – review & editing. MB: Conceptualization, Formal analysis, Supervision, Validation, Writing – review & editing. UD: Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1603759/full#supplementary-material>

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