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Investigation of in vitro susceptibility and resistance mechanisms to amikacin among diverse carbapenemase-producing *Enterobacteriaceae*

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Abstract

Objective This study aims to assess the in vitro drug susceptibility of various Carbapenemase-Producing *Enterobacteriaceae* (CPE) genotypes and elucidate the underlying mechanisms of amikacin resistance.

Methods A total of 72 unique CPE strains were collected from the Second Hospital of Jiaxing between 2019 and 2022, including 51 strains of *Klebsiella pneumoniae*, 11 strains of *Escherichia coli*, 6 strains of *Enterobacter cloacae*, 2 strains of *Klebsiella aerogenes*, 1 strain of *Citrobacter freundii*, and 1strain of *Citrobacter werkmanii*. Among these strains, 24 carried bla_{KPC} gene, 20 carried bla_{NDM} gene, 23 carried $bla_{OXA-48-like}$ gene, and 5 carried both bla_{KPC} and bla_{NDM}. We measured the in vitro activity of amikacin and other common antibiotics. Strains carrying bla_{OXA-48}-like gene were selected for whole genome sequencing (WGS) via next-generation sequencing to identify genes related to antimicrobial resistance (AMR) and virulence factor (VF).

Results Out of the 72 CPE strains tested, 41.7% exhibited resistance to amikacin. The drug resistance rates for *K. pneumoniae*, *E. coli*, and *Enterobacter* spp. were 51.0%, 27.3%, and 10.0%, respectively. The majority of the CPE strains (> 90%) displayed resistance to cephalosporins and carbapenems, while most of them were sensitive to polymyxin B and tigecycline (97.2% and 94.4%). The amikacin resistance rate was 100% for strains carrying bla_{OXA-48}, 20.8% for those with bla_{KPC}, 5.0% for those with bla_{NDM}, and 20.0% for those with both bla_{KPC} and bla_{NDM}. These differences were statistically significant ($P < 0.05$). Through sequencing, we detected aminoglycoside resistance genes *rmtF* and *aac(6')-Ib*, VF genes *iucABCD* and *rmpA2* in OXA-48-producing multidrug resistance and highly virulent strains. These genes were located on a IncFIB- and IncHI1B-type plasmid, respectively. Both plasmids were highly homologous to the plasmid from OXA-232 strains in Zhejiang province and Shanghai province. Integration of these resistance genes into the IncFIB plasmid, facilitated by the IS*6* and/or Tn3 transposons, resulted in OXA232-producing *K. pneumoniae* with amikacin resistance.

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Conclusion This study identified significant amikacin resistance in CPE strains, particularly in those carrying the bla_{OXA-48} gene. Resistance genes *rmtF* and *aac(6')-Ib* were identified on plasmids. These results highlight the need for careful monitoring of amikacin resistance.

Keywords Carbapenemase-producing Enterobacteriaceae, Drug resistance, Amikacin, Whole-genome sequencing

Introduction

The rise of antimicrobial resistance (AMR) among pathogenic bacteria presents a significant challenge to public health globally. Among the multidrug-resistant organisms, Carbapenemase-Producing *Enterobacteriaceae* (CPE) are particularly concerning due to their capacity to hydrolyze a broad spectrum of β-lactam antibiotics, including carbapenems, which are often considered the last line of defense against severe bacterial infections [1]. This resistance compromises treatment efficacy and limits therapeutic options, leading to increased morbidity and mortality [2].

Carbapenemase enzymes, such as *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), and oxacillinase-48 (OXA-48), are widely recognized contributors to the resistance phenotype in *Enterobacteriaceae*. The spread of these resistance genes, often facilitated by plasmids, poses a significant threat in both hospital and community settings [3]. While much attention has been focused on the resistance conferred by these enzymes against β-lactam antibiotics, the increasing prevalence of resistance to other critical antibiotic classes, such as aminoglycosides, has also become a pressing concern [4].

Amikacin, an aminoglycoside antibiotic, has been utilized as a treatment option for infections caused by multidrug-resistant *Enterobacteriaceae*, including CPE [5]. However, resistance to amikacin is emerging, further complicating the management of these infections [6]. The mechanisms underlying amikacin resistance in CPE are multifaceted, involving enzymatic modification, target site alterations, and efflux pump overexpression [7]. Given the already limited therapeutic options against CPE, understanding the specific mechanisms of resistance to amikacin within this context is crucial for informing effective treatment strategies.

This study aims to evaluate the in vitro drug susceptibility of various CPE genotypes and elucidate the underlying mechanisms of amikacin resistance. By analyzing 72 unique CPE strains collected from the Second Hospital of Jiaxing between 2019 and 2022, we seek to characterize the resistance patterns and identify key genetic factors contributing to amikacin resistance. Our findings will enhance the understanding of resistance mechanisms and support the development of targeted interventions to manage infections caused by CPE.

Materials and methods

Sample collection

A total of 72 unique CPE strains were collected from the Second Hospital of Jiaxing between 2019 and 2022. These strains consisted of 51 *Klebsiella pneumoniae*, 11 *E. coli*, 6 *Enterobacter cloacae*, 2 *Klebsiella aerogenes*, 1 *Citrobacter freundii*, and 1 *Citrobacter werkmanii*. The carbapenem-resistant strains (CRE) were identified according to the Clinical and Laboratory Standards Institute (CLSI) M100-S32 document, with a minimal inhibitory concentration (MIC) of imipenem ≥ 2.0 mg/L or meropenem ≥ 2.0 mg/L and ertapenem ≥ 0.5 mg/L [3]. Species identification was confirmed by MALDI-TOF/MS system. These CRE strains were isolated from various sources, including sputum (32, 44.4%), urine (21, 29.2%), bile (7, 9.7%), wound secretions (3, 4.2%), catheter drainage fluid (3, 4.2%), bronchoalveolar lavage fluid (2, 8.0%), blood (2, 8.0%), cerebrospinal fluid (1, 1.4%) and hydrothorax (1, 1.4%.). We identified carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}-like) in the CRE strains using multiplex PCR, followed by a preliminary screening of carbapenemase expression via colloidal gold immunochromatography. *Escherichia coli* ATCC 25,922 served as the quality control strain. This study was approved by the hospital ethics committee (Approval No: JXEY-2021SW044).

Instruments and reagents

The following instruments and reagents were exploited in our study: MALDI-TOF MS (Biomerieux, France); Phoenix M50 Automated microbial System (BD, USA); Carbapenemase detection kit (colloidal gold immunochromatography) (CARBA 5, Shanghai Fosun Diagnostics); Source of DNA extraction kit (Beijing Tiangen Biological Co., LTD.); Broth microdilution drug sensitivity reagent (Wenzhou Kangtai Biotechnology Co., LTD.); NovaSeqTM 6000 (Illumina, USA) and PacBio Sequel (PacBio, Inc.) were used for next-generation sequencing and third-generation sequencing, respectively.

Antimicrobial susceptibility testing

We conducted antimicrobial susceptibility testing (AST) using the broth microdilution method as recommended by the Clinical and Laboratory 64 Standards Institute (CLSI) [8]. We assessed the susceptibility of 72 CPE strains to a range of antimicrobial agents, including: cephalosporins (ceftazidime, ceftriaxone, cefepime), β-lactam/β-lactamase inhibitor combinations

(cefoperazone-sulbactam, piperacillin-tazobactam, ceftazidime-avibactam), carbapenems (ertapenem, imipenem, meropenem), monobactam (aztreonam), fluoroquinolones (ciprofloxacin, levofloxacin), folate metabolic pathway inhibitors 68 (sulfamethoxazole), aminoglycosides (amikacin and gentamicin), polymyxin B, and tigecycline. We determined the MICs for these 17 antimicrobial agents. The cefoperazone-sulbactam refers to the break point of cefoperazone, and for tigecycline, we used the MIC breakpoints for *Enterobacteriaceae* (susceptible, ≤2 mg/L; resistant, ≥8 mg/L) issued by the Food and Drug Administration as our reference points. Quality control strains for AST included *Escherichia coli* ATCC 25,922, *E. coli* ATCC 35,218 and *K. pneumoniae* ATCC 700,603.

Detection of carbapenemase gene by enzyme Immunochromatography

Three freshly isolated colonies were selected, resuspended in 100 μL of sample diluent, and added ten drops of this solution to the test wells. After 10 min, the presence or absence of bands was observed as an indication of carbapenem resistance.

Confirmation of carbapenemase genotype

The monoclonal CPE strain was cultured on solid LB medium at 37℃ for 24 h. A single colony was selected and incubated in LB liquid medium overnight at 37℃ and 220 rpm. A bacterial culture of 1 mL was centrifuged at 10,000 rpm for 1 min, and the supernatant was removed, leaving the bacterial pellet. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). For isolates showing non-susceptibility to carbapenems, we screened for common carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{IMP}) through PCR amplification using specific primers based on the work of Rehman MU (as shown in Table 1). The reaction system consisted of 20 μL: Premix TaqTM 6 μL, 1 μL each of upstream and downstream primers (50 μmol/L), 2 μL DNA template, and 2 μL sterile double-distilled water. The reaction conditions were as follows: 94 ℃ for 5 min; 94 ℃ for 30 s, 57 ℃ for 40 s, and 72 ℃ for 1 min, for 35 cycles; and 72 ℃ for 7 min.

Table 1 Primers used for carbapenemase detection

Genes	Primers	Sequences (5′-3′)	Product sizes (bp)
bla-$_{OXA-48\ like}$	OXA-48-F	GCGTGGTTAAGGATGaacAC	438
	OXA-48-F	CATCAAGTTCaacCCaacCG	
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	621
	NDM-F	CGGAATGGCTCATCACGATC	
bla_{KPC}	KPC-F	CGTCTAGTTCTGCTGTCTTG	798
	KPC-R	CTTGTCATCCTTGTTAGGCG	
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	390
	VIM-R	CGAATGCGCAGCACCAG	
bla_{IMP}	IMP-F	ATGAGCAAGTTATCTTAGTATTC	765
	IMP-R	GCTGCaacGACTTGTTAG	

Short-read and long-read whole genome sequencing

Upon the initial growth of transferred colonies over a span of two weeks, we scraped two rings of medium slant, placed them in a 1.5 ml centrifuge tube with 300 μl of deionized water, and subjected them to inactivation at 80 °C for 30 min.

For short-read sequencing, genomic DNA was extracted from the inactivated bacterial cells using the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop spectrophotometer and Qubit fluorometer, respectively. The DNA library was then prepared using the NEB Next Ultra II DNA Library Prep Kit for Illumina. The library preparation included fragmentation of the DNA to an average insert size of approximately 350 bp, followed by end repair, adapter ligation, and PCR enrichment. The libraries were sequenced on the Illumina NovaSeq 6000 platform, generating paired-end reads of 150 bp (PE150). The sequencing run produced a total of approximately 0.6 to 1.2 Gb of data per sample, with an average coverage depth of 100x. Raw reads were quality-trimmed using fastp v0.23.2, and the resulting clean reads were assembled with Unicycler v0.5.0 under default settings [9, 10].

For long-read sequencing, high molecular weight genomic DNA was isolated using the phenol-chloroform extraction method to ensure integrity. The DNA library for third-generation sequencing was prepared using the PacBio SMRTbell Express Template Prep Kit 2.0. The library was size-selected for fragments larger than 10 kb using the BluePippin system. Sequencing was performed on the PacBio Sequel platform, producing continuous long reads (CLR). On average, approximately 5 Gb of long-read data was generated per sample, with an N50 read length of around 15 kb. Raw PacBio long reads were quality-filtered using Filtlong v0.2.1 (https://github.com/rrwick/Filtlong). A hybrid assembly was then performed with the trimmed long and short reads using Unicycler v0.5.0 under default settings, producing a complete genome assembly.

Detection of aminoglycoside antimicrobial resistance genes

Assembly of sequencing raw data fragments was carried out using Unicycler v0.5.0 assembly software, while Prokka v1.14.6 software was utilized for genome annotation of the assembled results [10, 11]. RGI v5.2.1 software was employed for drug resistance gene functional annotation based on the CARD database [12]. The identification

of aminoglycoside resistance genes was conducted using the "strict" detection mode in RGI, with cutoff values of ≥95% identity and ≥80% coverage to ensure high confidence in gene detection.

Detection of mobile genetic elements

The assembled plasmid sequences were identified with the PLSDB database using the 'screen' command of Mash [13]. For plasmid screening, a maximum p-value of 1e-5 and a minimum identity of 90% were applied to ensure accurate identification while minimizing biases. The mobility and genotyping of plasmids were predicted by MOB-suite [14]. To compare the plasmids, we performed a megaBLAST search against the NCBI GenBank database. For other types of Mobile genetic elements (MGEs), Phigaro, ICEfinder, digIS, and IslandPath-DIOMB were utilized with default parameters to detect prophage, integrative and conjugative element (ICE), insertion sequences (IS), and genomic island (GI), correspondingly [15–18].

Statistical methods

The statistical analysis was performed using SPSS 18.0 software. Count data were expressed as rates, and group comparisons was analyzed using the χ2 test. Rank data were analyzed using the rank sum test (Wilcoxon two-sample comparison method). P-values less than 0.05 were considered statistically significant.

Results

Drug susceptibility testing of 72 CPE strains to amikacin

Among the enrolled 72 CPE strains, all were found to possess carbapenemase genes. Of these, 24 strains carried bla_{KPC}, 23 carried $bla_{OXA-48-like}$, 20 carried bla_{NDM}, and 5 strains carried both bla_{KPC} and bla_{NDM} genes (Supplementary Table 1). Each of these genes was expressed, confirming that all strains were capable of producing carbapenemases and contributing to the observed resistance profiles. When testing 72 CPE strains for their susceptibility to amikacin, we found that 40.3% of them were resistant to the drug (Table 2). Specifically, *K. pneumoniae* had the highest amikacin-resistance rate at 51.0%, followed by *Escherichia coli* at 27.3%, and *Enterobacter* spp. at 10.0%.

In vitro antimicrobial susceptibility

The majority of CPE strains (>90%) exhibited resistance to various antibiotics, including cephalosporins, cefoperazone-sulbactam, piperacillin-tazobactam, aztreonam, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, and carbapenems (Table 3). Among the isolates, 27.8% (20/72) were resistant to ceftazidime-avibactam, 41.7% (30/72) to amikacin, and 79.2% (57/72) to gentamicin. Remarkably, polymyxin B and tigecycline exhibited strong antibacterial activity against CPE strains, with sensitivity rates of 97.2% and 94.4%, respectively. In this set of 72 CPE strains, the amikacin resistance rate was influenced by the type of carbapenemase gene present. Specifically, bla_{OXA-48}-like carrying isolates showed a 100% resistance rate to amikacin, while for bla_{KPC}-carrying strains, the resistance rate was 20.8%. Among bla_{NDM}-carrying strains, the resistance rate was 5.0%, and for strains carrying both bla_{KPC} and bla_{NDM} genes, the resistance rate was 20.0%.

Prevalence of aminoglycoside resistance genes in bla_{OXA-48}-like strains

All 23 CPE strains carrying bla_{OXA-48} were identified as *K. pneumoniae*, and DNA sequencing results revealed that they were $bla_{OXA-232}$, ST15 clones. More importantly, all strains encode the 16 S rRNA methyltransferase *rmtF*, which confers high-level resistance to multiple aminoglycosides, and the aminoglycoside 6'-N-acetyltransferase *aac(6')-Ib*, which confers dual resistance to aminoglycosides and fluoroquinolones through its fluoroquinolone-acetylating activity.

Genetic context of plasmids carrying aminoglycoside resistance genes

We randomly selected 14 strains for whole genome sequencing using the Illumina platform. These strains were chosen to represent a range of antimicrobial resistance profiles and genetic backgrounds. One of these strains was then further sequenced using the PacBio platform. All strains were MDR *K. pneumonia* with multiple

Table 2 Antibacterial activity of amikacin against all strains (MICs, mg/L)

Species	Number of strains	MIC (mg/L)			Resistant strains	Susceptible strains	Resistance rate
		MIC50	MIC90	Range			
K. pneumoniae	51	64	1024	0.5–1024	26	25	51.0
E. coli	11	2	1024	1-1024	3	8	27.3
E. cloacae	6	2	2	0.5–64	1	5	16.7
K. aerogenes	2	/	/	1–2	0	2	0
Citrobacter spp*	2	/	/	1–2	0	2	0
Total	72	2	1024	0.5–1024	30	42	41.7

Note: *Citrobacter freundii* and *Citrobacter werkmanii*

Table 3 Susceptibility of strains carrying different β-lactamase genes to clinical commonly used antibiotics and amikacin (%)

Antibacterial agents	CRE($n=72$)		bla_{KPC} ($n=24$)	bla_{NDM} ($n=20$)	$bla_{OXA-48-like}$ ($n=23$)	$bla_{KPC+NDM}$ ($n=5$)
	MIC range	R%	R%	R%	R%	R%
Cefoperazone-sulbactam	32 ->128	91.7	95.8	93.1	100	100
Piperacillin-tazobactam	> 256	100	100	100	100	100
Ceftazidime-avibactam	0.5->32	27.8	0	100	0	100
Ceftazidime	2->32	98.6	95.8	100	100	100
Ceftriaxone	2->32	97.2	91.7	100	100	100
Cefepime	2->32	95.8	87.5	100	100	100
Aztreonam	2->128	94.4	95.8	90	100	80
Ertapenem	8->32	100	100	100	100	100
Imipenem	2->16	95.8	100	100	91.3	80
Meropenem	4->16	100	100	100	100	100
Amikacin	0.5–1024	41.7	20.8*	5*	100*	20*
Gentamycin	1->128	77.8	87.5	45	100	60
Ciprofloxacin	0.5->8	95.8	100	85	100	100
Levofloxacin	0.5->16	88.9	83.3	80	100	100
Trimethoprim-sulfamethoxazole	0.5->32	93.1	87.5	90	100	100
Polymyxin B	0.25->8	2.8	4.2	0	4.3	0
Tigecycline	0.125-4	5.6	4.2	10	0	20

Note: CRE, carbapenem-resistant *Enterobacteriaceae*; MIC50/90, 50%/90% minimum inhibitory concentration; %R, % of isolates resistant; %S, % of isolates susceptible

*The resistance rate to amikacin in bla_{KPC}, bla_{NDM}, $bla_{KPC+NDM}$ groups was statistically different from that of $bla_{OXA-48-like}$ group (Chi-squared test, $P<0.05$)

Table 4 Plasmids with high homology with plas2 in NCBI database

Species	Strain	MLST	Plasmid	Identity (%)	Coverage (%)	Accession	Area	Date	AMR
K. pneumoniae	KPTCM	ST15	pKPTCM-2	100	100	CP097387.1	Hangzhou, China	2022	*aac(6')-Ib*, *rmtF*
K. pneumoniae	KP3295	ST15	pKP3295-3	100	100	CP079728.1	Yiwu, China	2018	*aac(6')-Ib*, *rmtF*
K. pneumoniae	WSD411	ST15	pWSD411_4	100	100	CP045677.1	Hangzhou, China	2018	*aac(6')-Ib*, *rmtF*
K. pneumoniae	RJKP36	ST15	pRJKP36-2	100	100	CP100078.1	Shanghai, China	2018	*aac(6')-Ib*, *rmtF*
K. pneumoniae	Kp7450	ST15	p2	100	100	CP090470.1	Shanghai, China	2020	*aac(6')-Ib*, *rmtF*

AMR genes including *gryA* mutations, *rmtF*, *aac(6')-Ib*, $bla_{CTX-M-15}$, *SHV-28*, *arr-2*, and $bla_{OXA-232}$. An average number of AMR genes among these strains was 40 (between 39 and 44). Plasmid profiling revealed that all these strains had the same plasmid pattern. In addition, we successfully obtained the complete genome sequence of one representative strain through hybrid assembly. We identified a IncFIB-type plasmid (plas2) within the complete genome carrying the *aac(6')-Ib* and *rmtF* genes. Plas2 was classified as an IncFIB-type plasmid, which was non-mobilizable for lacking relaxase, mate-pair formation and *oriT* sequences. Online BLASTn analysis of the NCBI nucleic acid database revealed five highly homologous plasmids for plas2 sequences, as shown in Table 4. The results demonstrated that most of these plasmids were derived from ST15 *K. pneumoniae* strains, with only one plasmid being isolated from *K. pneumoniae* strain 47,733 of ST147. The earliest isolation of these plasmids' dates back to 2011.

Within the plasmid, a genomic island (GI) sequence spanning 8,553 base pairs was identified, encompassing both the *aac(6')-Ib* and *rmtF* genes. Furthermore, insertion sequence (IS) analysis unveiled the presence of an IS*6* family transposon (IS*6100*), and its end was immediately followed by a Tn3 family transposon (ISXc4). To compare plas2 with other plasmids, whole-genome alignments were conducted for pWSD411_4, p47733_IncFIB, p1605752FIB_2, and pPMK1-B, and the results were visualized using BRIG software [19]. The locations of antimicrobial resistance genes and IS transposons were distinctly marked, as illustrated in Fig. 1. The findings revealed that, except for plas2 and pWSD411_4, the remaining three IncFIB-type plasmids previously isolated from *K. pneumoniae* lacked the sequence segment encompassing the GI.

Convergence of virulence and resistance in a IncF-type plasmid

An average number of VFs among 14 genomes were 51.8 (between 50 and 52). Notably, all sequenced strains were hypervirulent for carrying aerobactin operons *iucABCD* and an activator for capsular polysaccharide (CPS) synthesis *rmpA2*, as aerobactin is specifically associated with growth in blood and is a stronger predictor of the hypervirulence phenotype. The genomic localization analysis revealed these VF genes were located on a IncHI1B-type plasmid (plas1). After the online BLAST search of the NCBI database, we retrieved seven homologous

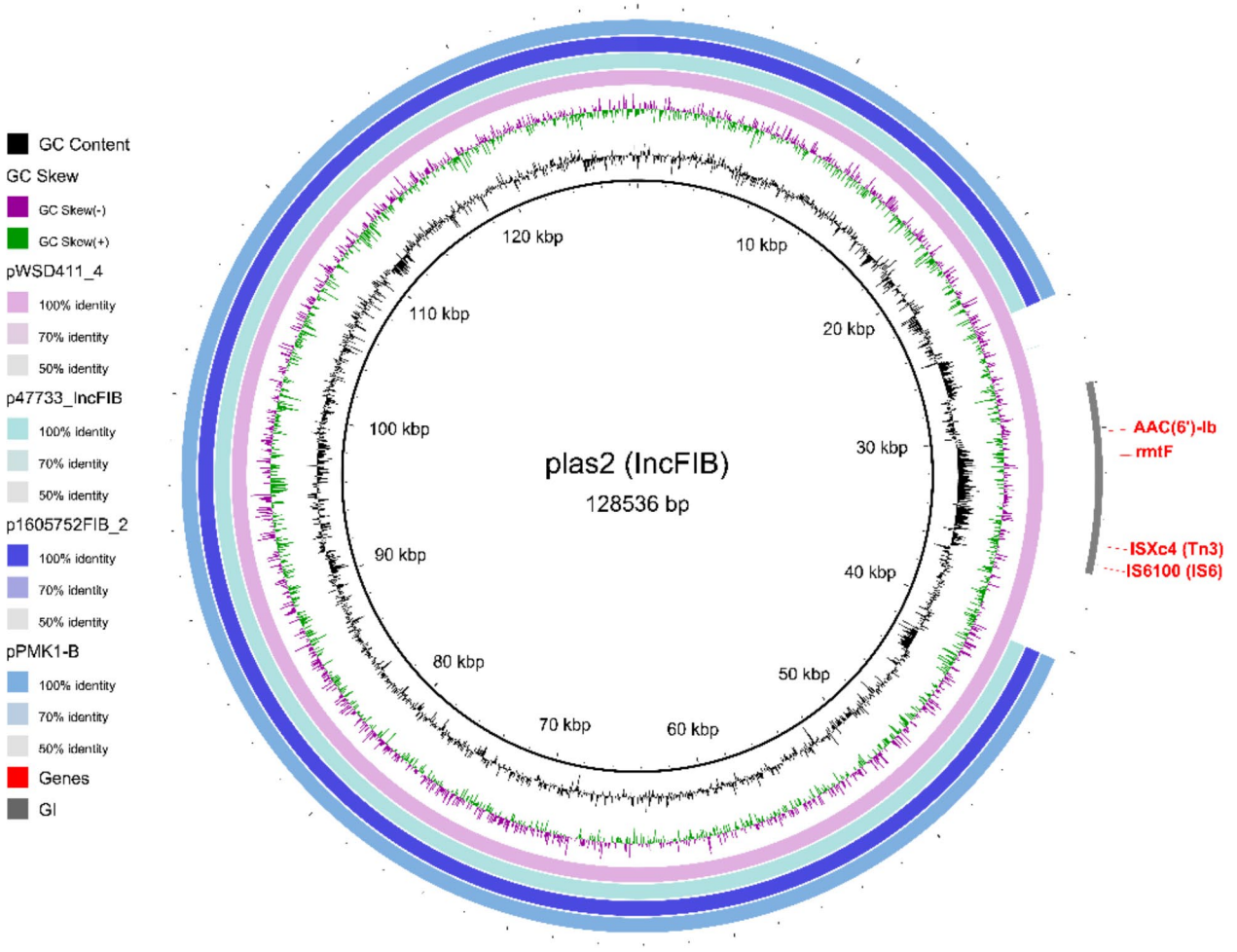

Fig. 1 Genome comparison circle of plas2 and its homologous plasmids
Note: The inner circle represents the position coordinate of the plas2 plasmid genome sequence. From inside to outside: Genomic GC content; 2. Genomic GC Skew value; 3. Comparison of pWSD411_4, p47733_IncFIB, p1605752FIB_2 and pPMK1-B with plas2 plasmid; 4. Location of resistance genes aac(6')-Ib and *rmtF*, transposons IS6100 and ISXc4 on plas2 plasmid genome

plasmids. Despite the heterogeneity observed in these plasmids, using plas1 as the reference sequence, the sequence coverage varies from 53 to 99% (Fig. 2). However, the average sequence identity and coverage for *iucABCD* and *rmpA2* are 95.3% and 99%, respectively. In addition, this VF plasmid was predicted to be non-mobilizable for lacking relaxase, mate-pair formation and *oriT* sequences.

Discussion

Carbapenemase production is the most common resistance mechanism of *Enterobacteriaceae* bacteria against carbapenems [20]. In clinical settings, the most commonly encountered carbapenemase belong to three Ambler classes: Ambler A serine hydrolases (such as KPC), Ambler B metalloenzymes (such as NDM), and Ambler D serine hydrolases (such as OXA-48). This group of data and related literatures suggested that the CPE had a high resistance rate to commonly used antibiotics in the clinic [21, 22]. This study showed that most of the CPE strains (>90%) were resistant to cephalosporins, cefoperazone sulbactam, piperacillin-tazobactam, aztreonam, ciprofloxacin, trimethoprim-sulfamethoxazole, and carbapenems. Interestingly, 72.2% and 58.3% strains were susceptible to ceftazidime-avibactam and amikacin, respectively. However, aminoglycoside susceptibility varied among different CPE isolates. For instance, KPC-2-producing *K. pneumoniae* strains isolated in Taiwan, China, exhibited a 69% resistance rate to amikacin, while *E. coli* strains showed only 3% resistance rate [23]. Our study results indicate a 41.7% resistance rate of 72 CPE strains to amikacin (with *K. pneumoniae* demonstrating a resistance rate of 51.0%, and *E. coli* demonstrating a resistance rate of 27.3%).These findings are consistent

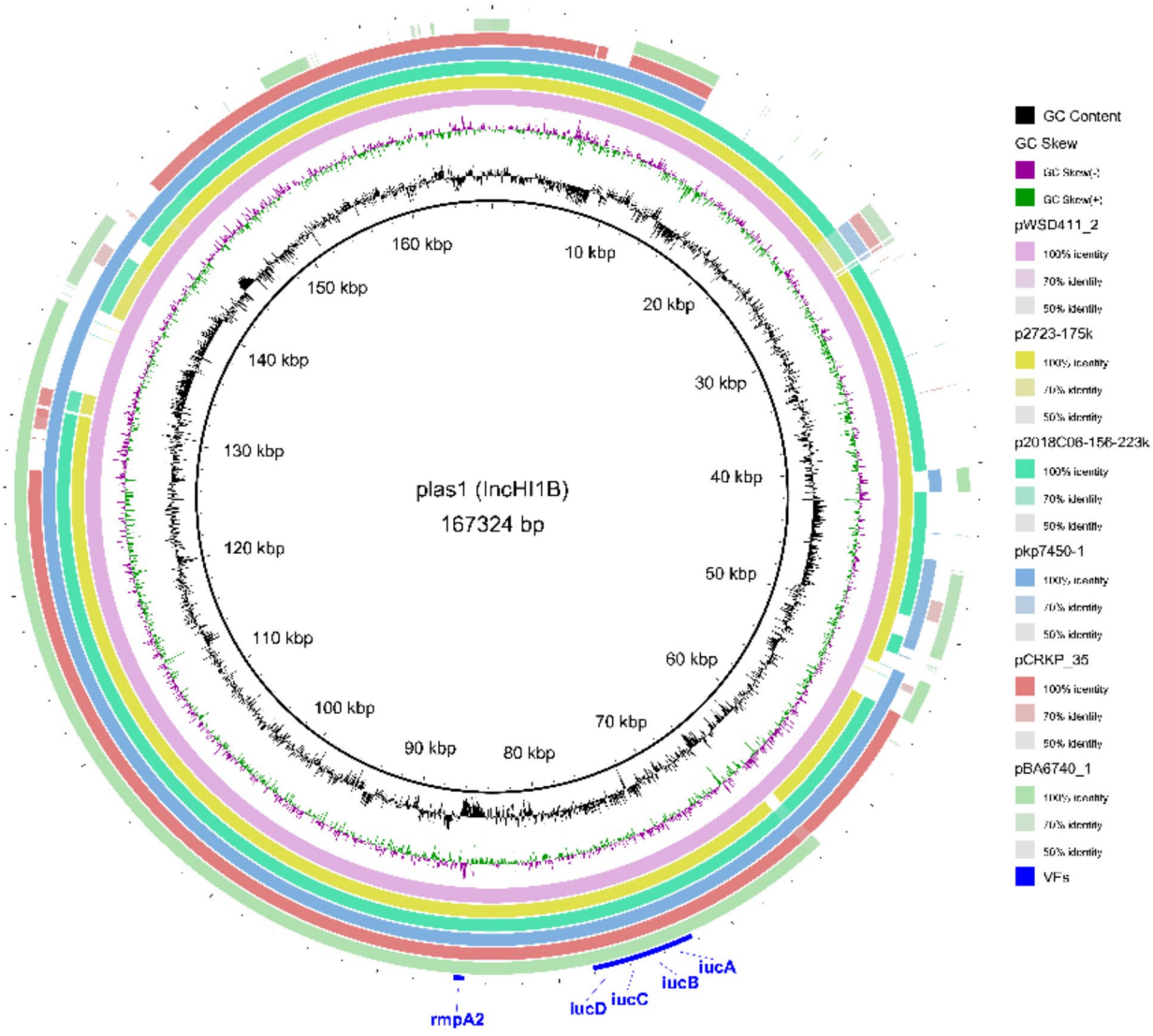

Fig. 2 Genome comparison circle of IncHI1B-type plas1 and its homologous plasmids
Note: The inner circle represents the position coordinate of the plas1 plasmid genome sequence. From inside to outside: Genomic GC content; 2. Genomic GC Skew value; 3. Comparison of pWSD411_2, p2723-175k, p2018C06-156-223k, pkp7450-1, pCRKP_35 and pBA6740_1 with plas2 ; 4. Location of VF genes *iucABCD* and *rmpA2* on plas1 plasmid genome

with a domestic report by Hu Fumin et al., which demonstrated resistance rates of 49.6% [24].

Table 3 indicates that the drug susceptibility of Carbapenemase-producing Enterobacteriaceae strains carrying different carbapenemase-encoding genes to amikacin varies significantly in vitro. Avibactam in ceftazidime-avibactam has a wide range of inhibitory activities against B-lactamases such as various class A enzymes (such as CTX-M-15, KPC-2, etc.), class C enzymes (*AmpC*) and some class D enzymes (such as OXA-48). The resistance rate of $bla_{\mathrm{OXA-48}}$-like carrying *K. pneumoniae* to amikacin was 100%, and it cannot inhibit the B-type metalloenzyme (NDM-1), which leads to ceftazidime-avelbactam resistance to the CPE carrying bla_{NDM} [25]. It's worth noticing though, amikacin resistance in $bla_{\mathrm{OXA-48}}$-like *K. pneumoniae* was 100%, with extremely high resistance levels (MIC values ≥ 1024ug/mL), consistent with the results reported by Han R [24]. In this study, WGS showed that all strains carried the *aac(6')-Ib* aminoglycoside modifying enzyme and 16 S rRNA methylase gene *rmtF*. However, only 5% of the 20 *Enterobacteriaceae* strains carrying the bla_{NDM} genotype were resistant to amikacin, with MIC values ranging from 0.5 to 32ug/mL. *aac(6')-Ib* and *rmtF* genes were not further detected in the amikacin-resistant *E. coli* strains.

In this study, we observed that CPE strains carrying bla_{OXA-48} exhibited an extremely high level of resistance to amikacin, with both the MIC50 and MIC90 exceeding 1024 ug/mL. The *aac(6')-Ib* gene encodes an acetyltransferase enzyme that modifies aminoglycosides, including amikacin, by adding an acetyl group. This modification impairs the antibiotic's ability to bind to bacterial ribosomes, thereby reducing its effectiveness. The *rmtF* methylase gene encodes a methyltransferase enzyme that specifically methylates the 16 S rRNA within the ribosomal RNA, preventing the binding of aminoglycosides like amikacin. Both of these mechanisms contribute to the resistance of bacteria to aminoglycosides by altering the drug's target site or modifying the drug itself [26].

Ten groups of methylases have been found, including *armA*, *RmtABCDEFGH*, *npmA*. Among those, *armA* is dominant in Europe, *armA* and *RmtB* are dominant in 191 North America, and *RmtD* is dominant in Latin America [26]; In China, *armA* and *RmtB* are the main methylases. However, the presence of *rmtF*, as a new member of the aminoglycoside 16 S rRNA N7 G1405 methyltransferase family, was quite rare in China previously. In fact, recent reports have shown its emergence, often in conjunction with the $bla_{OXA-232}$ carbapenemase gene [27, 28]. Importantly, the prevalence of *rmtF* is significantly associated with carbapenemase genotypes and ST clones of strains in different regions. For instance, *rmtF* is mainly isolated from *Enterobacteriaceae* bacteria producing bla_{NDM}-type carbapenemase in India and the United Kingdom, while in Switzerland, it is mainly isolated from CPE strains producing bla_{KPC-2} and $bla_{OXA-232}$, with ST231 as the dominant clone [29].

The identification of the IncFIB-type plasmid (plas2) carrying the *aac(6')-Ib* and *rmtF* genes has significant clinical implications, particularly in the context of treatment strategies for infections caused by multidrug-resistant *K. pneumoniae*. The presence of these resistance genes within a non-mobilizable plasmid suggests a potential for stable inheritance within bacterial populations, which could lead to persistent resistance issues in clinical settings [30]. Understanding the plasmid's genomic features, such as the presence of GIs and transposons, allows for better prediction of gene transfer events and the spread of resistance. This knowledge could inform targeted therapeutic interventions, such as the development of inhibitors that block the activity of these specific resistance genes or the use of combination therapies to overcome the resistance conferred by these plasmids [31]. Moreover, monitoring the spread of such plasmids in clinical isolates could help in tailoring antibiotic stewardship programs and improving patient outcomes by preventing the dissemination of highly resistant strains [32].

This study revealed the presence of both virulence and AMR determinants on two F-type plasmids. Beisde, amidoglycoside resistance genes *aac(6')-Ib* and *rmtF* were found only in the plasmids from China. In terms of time, aminoglycoside resistance genes were only found in the plasmid after 2018, and it was preliminarily speculated that the plasmid was exogenously acquired. Further detection of mobile genetic elements suggested that these two aminoglycoside resistance genes might have been acquired from *K. pneumoniae* and integrated into the IncFIB plasmid through IS sequences. Since the IncFIB plasmid is non-mobilizable and based on the data from the NCBI nucleic acid database, there is no evidence that the aminoglycoside resistance phenotype of *K. pneumoniae* strains isolated in this project is caused by the transfer of plasmids carrying these resistance genes. These findings suspected that there is a clone of ST15 MDR and hypervirulent *K. pneumoniae* that has recently spread aminoglycoside resistance in eastern China. Therefore, attention should be paid to the clonal spread of strains from regions outside the local area in clinical practice.

Conclusion

The epidemic strains of CPE associated with different types of carbapenemase vary from one country or region to another, and their resistance to amikacin also differs. In clinical practice, monitoring amikacin resistance involves assessing drug resistance phenotype, determining MIC values, and identifying specific resistance genes. Additionally, plasmid analysis serves as an effective complementary tool for medical institutions to efficiently screen drug resistance genes and understand their potential for transmission. Based on these findings, appropriate monitoring strategies and infection control measures can be implemented to prevent the occurrence of nosocomial (hospital-acquired) infections. This proactive approach is crucial for addressing the evolving landscape of antibiotic resistance and safeguarding patient safety.

Supplementary Information

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Supplementary Material 1

Author contributions

XS.L and C.F conceived of the presented idea. X.W, J.Y and M.S carried out the experiment. XC.L prepared figures. X.W and XS.L wrote the main manuscript text. All authors reviewed the manuscript.

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Data availability
Genome sequences of all 14 strains have been deposited in the NCBI Genbank database under BioProject accession no. PRJNA975316.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethical Committee of the Second Affiliated Hospital of Jiaxing University. Informed consent was obtained from all of the participants involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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