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High-level ceftazidime-avibactam resistance by in-host evolution of bla_{KPC} genes: Emergence of a novel $bla_{KPC-102}$ variant and increase of bla_{KPC-33} copy number in *Klebsiella pneumoniae* strains from a lung transplantation recipient

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ABSTRACT

Objective: KPC-producing *Klebsiella pneumoniae* (Kp) infections have become one of the major threats to public health in China. Recently, the emergence of ceftazidime-avibactam (CZA) resistance due to the bla_{KPC} mutations has been increasingly reported.
Methods: Two CZA-susceptible Kp strains (Kp36854 carrying bla_{KPC-2} and Kp37523 without bla_{KPC}) and two CZA-resistant Kp strains (Kp38935 carrying double copies of bla_{KPC-33} and Kp38097 carrying a newly identified $bla_{KPC-102}$ gene) were isolated from a lung-transplanted patient during CZA treatment. This study analyzed the within-host evolutionary dynamics of bla_{KPC} in KPC-Kp strains.
Results: Compared with KPC-2, KPC-33 possessed only a D179Y substitution while KPC-102 harbored both D179Y and Y241D substitutions. We constructed KPC-δ33 with only a Y241D substitution, which means the correction of the first mutation that is also present in KPC-33 (D179Y). Cloning and expression experiments showed that CZA-MIC value of *E. coli* DH5α/pKPC102 was 512 mg/L, while CZA-MIC values of both *E. coli* DH5α/pKPC-33 and *E. coli* DH5α/pKPC-δ33 were 32/4 mg/L. Enzymatic kinetic analysis revealed that KPC-2 demonstrated the highest catalytic efficiency to nitrocefin and meropenem, whereas KPC-33 displayed higher catalytic efficiency against ceftazidime compared to other tested KPC variants. Regarding avibactam, KPC-2 showed the highest sensitivity, while KPC-δ33 and KPC-102 were less sensitive.
Conclusion: High-level CZA resistance was mediated by the novel $bla_{KPC-102}$ and double copies of bla_{KPC-33} gene, respectively, in two porin-deficient Kp strains from one patient. The CZA resistance resulting from bla_{KPC} mutation could be selected and evolved to be more diverse and heterogeneous within the host after CZA therapy. It is very essential to perform the surveillance of CZA-resistance for clinicians during treatment.

1. Introduction

Klebsiella pneumoniae carbapenemase (KPC)-producing *K. pneumoniae* (KPC-Kp) has been increasingly highlighted as a major threat to global public health due to the high mortality rates and huge financial burden of infections [1–4]. In China, Kp sequence type 11 (ST11) has been recognised as the dominant clone re-

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sponsible for KPC dissemination [4,5]. Ceftazidime-avibactam (CZA) is a β-lactam/β-lactamase inhibitor (BL/BLI) combination which was approved by the FDA in 2015 for complicated intra-abdominal infections, complicated urinary tract infections, hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) clinical practice [6,7]. This novel BL/BLI has demonstrated activity against common Gram-negative HAP/VAP pathogens, including certain drug-resistant (ESBL- and KPC-producing) *Enterobacterales* and MDR *Pseudomonas aeruginosa* strains. The recommended duration of therapy for FDA-approved indications is 5 to 14 days for complicated intra-abdominal infections and 7 to 14 days for complicated urinary tract infections and HAP/VAP [8,9].

The emergence of CZA resistance in KPC-Kp isolates has been increasingly reported with its extensive usage [10–15]. Notably, mutations in the KPC enzyme have been demonstrated to mainly contribute to the CZA resistance [10,12–14,16–20]. To date, 230 KPC variants have been identified worldwide (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/KPC). One or more amino acid mutations on loops (Ω-loop 164-179, loop 237-243 and loop 267–275) in the KPC variants were responsible for the CZA resistance [21].

In the present study, a total of 11 Kp isolates were recovered successively from bronchoalveolar lavage fluid (BALF) samples after transplantation. Among these, four representative Kp isolates were selected for further investigation based on their antimicrobial resistance phenotypes: two CZA-susceptible strains (Kp36854 and Kp37523) and two CZA-resistant strains (Kp38097 and Kp38935). One CZA-resistant Kp carried a newly-designated $bla_{\text{KPC-102}}$ gene, whereas the other harboured an increased copy number of $bla_{\text{KPC-33}}$ gene. The resistance profiles and mechanisms in these strains evolved within this host were further analysed. This study will help understand the complexity of the in-host evolution of bla_{KPC} genes in KPC-Kp strains and how they tried to survive under the pressure of CZA.

2. Materials and methods

2.1. Patient and clinical isolates

A 27-year-old female patient, with a history of acute myeloid leukaemia, allogeneic hematopoietic stem cell transplantation, chronic graft-versus-host disease, and bronchiolitis obliterans syndrome, failed to respond to standard medical therapies. On March 29, 2021, she underwent double lung transplantation. This patient had a complex series of antimicrobial changes based on stewardship principles (e.g. patient symptoms and susceptibility test results) since she had recurrent postoperative infections. A total of 11 Kp isolates were recovered successively from her BALF samples. These bacterial strains were identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik, Bremen, Germany) microflex LT/SH. Of these, four representative Kp isolates, including two CZA-susceptible strains (Kp36854 and Kp37523) and two CZA-resistant variants (Kp38097 and Kp38935), were selected for further investigation based on their changing antimicrobial resistance phenotypes. Kp36854 (KPC2-producing) was identified in mixed cultures with *Pseudomonas aeruginosa* and *Corynebacterium striatum* at day 14 post-transplant. Kp37523 (KPC-non-producing) was identified in mixed cultures with *Acinetobacter baumannii* at day 43 post-transplant. Both Kp38097 (KPC102-producing) and Kp38935 (KPC33-producing) were detected in mixed cultures with *P. aeruginosa* at day 70 and 100 post-transplant, respectively. The antibiotic treatment of this patient and the related four Kp strains are outlined in Fig. 1. In this patient, the same clones of Kp36854 and Kp38935, along with their related strains (Kp39469, Kp39633-2, Kp40062, Kp40977, and Kp49046), were repeatedly isolated. In contrast, the clones of Kp37523 and Kp38097 were not detected in subsequent samples. This study was approved by the Clinical Research Ethics Committee, China-Japan Friendship Hospital (2022-KY-054). The study was conducted in accordance with the ethical principles of the Clinical Research, China-Japan Friendship Hospital.

2.2. Phenotypic characterisation of the clinical isolates

Antimicrobial susceptibility tests (AST) were performed using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. Antimicrobial agents used for antimicrobial susceptibility testing included imipenem (IPM), meropenem (MEM), ceftazidime (CAZ), ceftazidime-avibactam (CZA), piperacillin-tazobactam (TZP), cefepime (FEP), aztreonam (ATM), amikacin (AK), tobramycin (TOB), ciprofloxacin (CIP), polymyxin B (PB), tigecycline (TGC) and levofloxacin (LEV). *E. coli* ATCC 25922 was used as the reference strain. All tests were conducted in triplicate, including three biological replicates in each test. The MIC values were interpreted by CLSI guidelines [22]. The efflux pump inhibitor (25 mg/L phenylarginine-β-naphthylamide, PAβN) and porin inhibitor (10 mg/L polymyxin B nonapeptide, PBMN) were added into broth microdilution to clarify the influence of the efflux pump and porin on CZA susceptibility, respectively.

2.3. Whole-genome sequencing (WGS) and bioinformatic analysis

WGS of all bacterial isolates was performed using both the HiSeq sequencer (Illumina) and Oxford Nanopore sequencing by Biohuge Co., Beijing, China. Short NGS reads and long nanopore reads were filtered to remove low-quality sequences and adaptors using Skewer and PoreChop (https://github.com/rrwick/Porechop), respectively. FASTQ format files of each sample were independently hybrid assembled using Unicycler v0.4.8. National Centre for Biotechnology Information Bacterial Antimicrobial Resistance Reference Gene Database (http://genepi.food.dtu.dk/resfinder) was used to search for potential matches applying the criteria of 90% identity and 60% minimum coverage length to obtain the acquired antimicrobial resistance genes. Gene annotation was carried out using Prokka1.12 [23]. In addition, the sequences of *ompk35* and *ompk36* were extracted from the assembled files and aligned with reference strain Kp NTUH-K2044 using SnapGene software version 3.2.1 (from Insightful Science; available at https://snapgene.com). Multilocus sequence typing (MLST) and Inc-type plasmids were obtained by MLST 2.0 servers and the Plasmid Finder 2.1 at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). The single-nucleotide polymorphism (SNP) was called using Snippy. Multiple sequence alignment (MSA) and locally collinear blocks (LCBs) analysis of Kp36854, Kp37523, and Kp38097 genomes were performed using the progressive Mauve software. LCB is a homologous region of sequence shared by two or more genomes.

2.4. Cloning experiments for bla_{KPC-2}, bla_{KPC-33} and $bla_{KPC-102}$

We constructed the KPC-δ33 with only a Y241D substitution outside of the Ω loop. The gene sequences of $bla_{\text{KPC-2}}$, $bla_{\text{KPC-33}}$, $bla_{\text{KPC-}\delta33}$ and $bla_{\text{KPC-102}}$ were amplified, and the purified PCR products were cloned into the pEASY-Blunt vectors (Transgen Biotech Co., China). The recombinant plasmids pKPC-2, pKPC-33, pKPC-δ33 and pKPC-102 were subsequently introduced into the *E. coli* DH5α strains by chemical transformation experiments. Transformants were selected on Luria Bertani (LB) agar plates with ampicillin (100 mg/L) and verified for the bla_{KPC} presence [24].

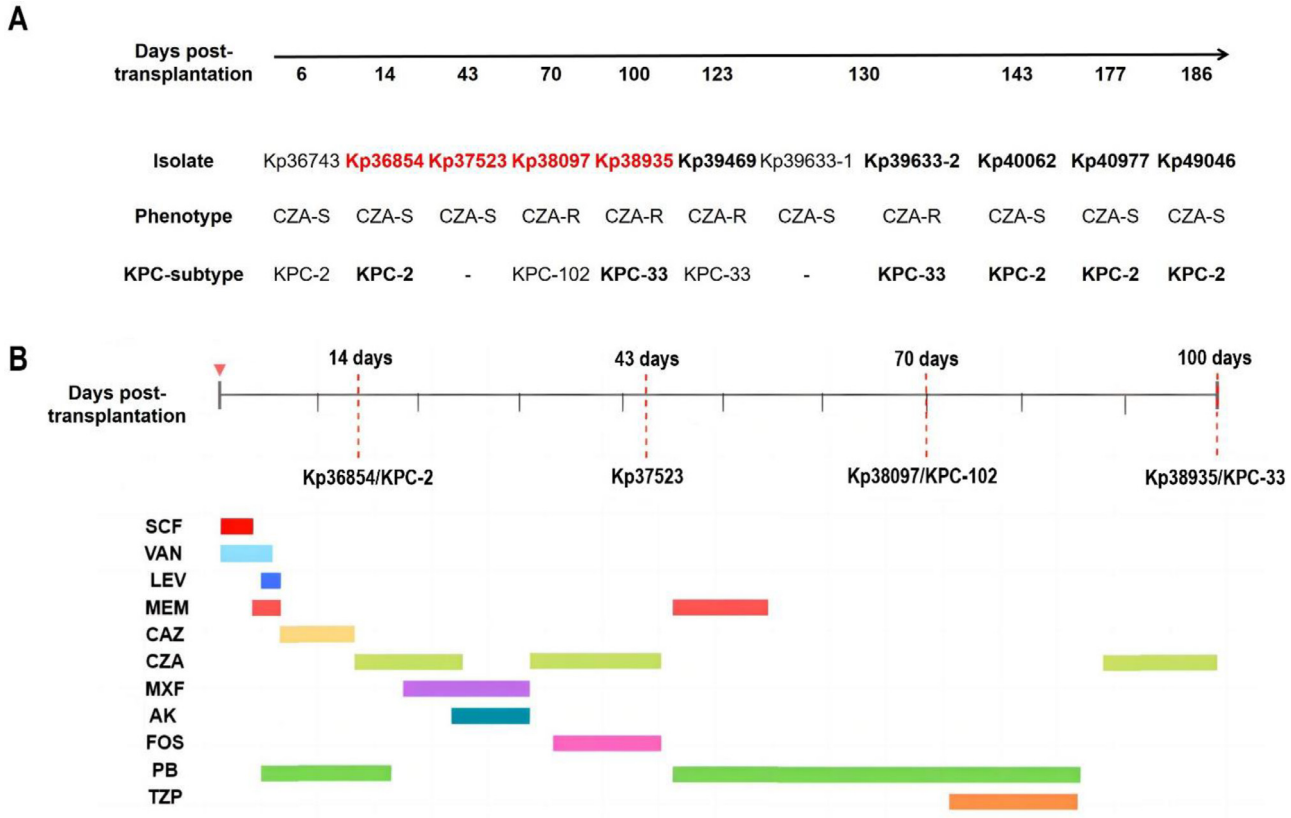

Fig. 1. (A) Description of isolation time, phenotypic characteristics, and KPC features of the 11 Kp strains. (B) Medical history of the patient. Four Kp isolates with different antimicrobial phenotypes were recovered successively from bronchoalveolar lavage samples after transplantation, including two CZA-susceptible strains (Kp36854 and Kp37523) and two CZA-resistant strains (Kp38097 and Kp38935). Different coloured bars in antibiotic treatment represent different antibiotics the patients were prescribed in the hospital. IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; CZA, ceftazidime-avibactam; TZP, piperacillin-tazobactam; FEP, cefepime; ATM, aztreonam; AK, amikacin; TOB, Tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; PB, polymyxin B.

2.5. Kinetic parameters of purified β-lactamases KPC variants

Enzyme kinetics were determined as previously mentioned [24]. The gene segments of the different KPC variants were amplified and cloned into the pET-28a (+) vector and then transformed into *E. coli* BL21 (DE3) competent cells. The *E. coli* BL21 (DE3) was induced by isopropyl beta-d-1-thiogalactopyranoside (IPTG) and the protein was purified by nickel affinity chromatography. Kinetic parameters were measured using a spectrophotometer at room temperature, and the data were fitted to the Michaelis-Menten equation *via* GraphPad Prism 8. The data for nitrocefin, meropenem and ceftazidime were restored by measuring the initial velocities at a variety of nitrocefin, meropenem and ceftazidime concentrations at 482 nm, 260 nm and 297 nm, respectively. For avibactam, the proteins were mixed with different concentrations of avibactam for 10 min, and then 80-μM nitrocefin was added as substrate. The absorbance at 482 nm was measured. The K_i and IC_{50} were calculated using GraphPad Prism 8.

2.6. Structure modelling of carbapenemase KPC-102

Homology modelling of carbapenemase KPC-102 was performed using the template of KPC-2 (PDB ID: 4ZBE) on the Swiss-Model workspace. The crystal structures of KPC-2 and KPC-102 were visualised by PyMOL Molecular Graphs System.

2.7. Carbapenemase detection

Different carbapenem enzyme detection methods were performed for Kp36854, Kp38097 and Kp38935. GeneXpert Carba-R (Cepheid, France, immunochromatographic method), Goldstream Carbapenem-resistant K.N.I.V.O. Detection K-Set (Beijing Gold Mountain River Tech Development Co. Ltd., Beijing, China, immunochromatographic method) and NG-test Carba 5 (NG Biotech, France, molecular testing) were used for detecting carbapenemase in these strains carrying bla_{KPC} gene.

2.8. Fitness evaluation

The growth curves of the isolated strains and constructed strains (*E. coli* DH5α/pKPC2, *E. coli* DH5α/pKPC33, *E. coli* DH5α/pKPCδ33 and *E. coli* DH5α/pKPC102) were developed and analysed to assess the fitness effects. Three independent cultures of each strain were grown overnight in LB broth. These cultures were diluted 1:100, and 200 μL of diluted culture was added into a flat-bottom 100-well plate in three replicates at 37°C with shaking. The optical density (OD) at 600 nm of each culture was measured every 2.5 minutes on average over a 24-hour period.

2.9. Conjugation experiments

Azide resistance was induced in the Kp strain (Kp700603), which was used as a recipient for conjugation experiments. Conjugation experiments were performed using Kp700603 (azide-resistant) as the recipient and Kp36835, Kp38097 and Kp38935 as the donors, respectively, as we described before [27]. Selection was made using LB agar plates containing ceftazidime (32 mg/L) and sodium azide (200 mg/L).

3. RESULTS

3.1. Basic characterisation of four clinical strains from one patient

AST showed that they were non-susceptible to TZP, FEP, ATM, TOB, CIP, LEV, CAZ. Kp36854 was susceptible to AK, CZA, PB and TGC, but resistant to IPM and MEM. Kp37523 was susceptible to IPM, MEM and CZA, but resistant to AK, PB and TGC. While Kp38097 and Kp38935 exhibited high-level CZA resistance, but increased IPM and MEM susceptibility compared to Kp36854 (Table 1).

All four strains belonged to ST11 based on MLST obtained from WGS data. Pairwise analysis of these strains revealed that the whole genomes between Kp36854 and Kp38935 differed only by a few SNPs (n=5), revealing they belonged to the same clone. While Kp36854, Kp37523 and Kp38097 belonged to different clones, which differed by more than 1000 SNPs.

3.2. High-level CZA resistance mediated by the novel bla$_{KPC-102}$ gene identified in Kp38097

Notably, the comparison analysis of WGS revealed that the *bla*$_{KPC}$ gene of Kp38097 contained two mutations in contrast to the *bla*$_{KPC-2}$ gene of Kp36854. It was designated *bla*$_{KPC-102}$, harbouring mutated nucleotides (G to T) at positions 535 and (T to G) at positions 721, which resulted in a variant with mutated amino acids (Asp-Tyr) at amino acid sequence position 179 and (Tyr-Asp) at amino acid sequence position 241 (Fig. 2). Cloning and expression experiments were performed to clarify the relationship between CZA resistance phenotype and *bla*$_{KPC}$ gene mutations. As expected, the CZA MIC values of *E. coli* DH5α/pKPC-102 carrying *bla*$_{KPC-102}$, *E. coli* DH5α/pKPC-33 carrying *bla*$_{KPC-33}$ and *E. coli* DH5α/pKPC-δ33 carrying *bla*$_{KPC-\delta33}$ were 512/4 mg/L, 32 mg/L and 32 mg/L respectively, by contrast, the CZA MIC of *E. coli* DH5α/pKPC-2 carrying *bla*$_{KPC-2}$ was 0.125/4 mg/L (Table 1). Moreover, the CZA MIC values of wild-type *E. coli* DH5α and the empty-vector-carrying strain *E. coli* DH5α/pEasy-Blunt E2 were less than 0.032 mg/L. Overall, these data demonstrated that the *bla*$_{KPC-102}$ could contribute to a huge increase in the CZA MIC value. Meantime, similar to *bla*$_{KPC-33}$, this resistant gene also conferred susceptibility to IPM and MEM (Table 1).

3.3. Increased copy number of bla$_{KPC-33}$ gene in plasmid pKP38935_2 originated from bla$_{KPC-2}$ in plasmid pKP36854_3

Furthermore, double copies of *bla*$_{KPC-33}$ gene in Kp38935 were identified by WGS analysis and they were located on a plasmid (pKP38935_2) (Fig. 3A). Meantime, the relative expression levels of the *bla*$_{KPC-33}$ gene in Kp38935 could reach 2.0-fold higher than that of the *bla*$_{KPC-2}$ gene in Kp36854, respectively (Fig. S1). Both *bla*$_{KPC-2}$-carrying pKP36854_3 in Kp36854 and *bla*$_{KPC-33}$-carrying pKP38935_2 in Kp38935 belonged to the IncFII (pHN7A8) incompatibility (Inc) groups. Another segment of the pKP38935_2 plasmid was formed through the transposition recombination of a partial sequence of pKP36854_3 (Fig. 3A). These findings indicated that the plasmid pKP38935_2 in Kp38935 might have originated from the plasmid pKP36854_3 in Kp36854.

3.4. Genetic environment analysis of bla$_{KPC-102}$, bla$_{KPC-2}$ and bla$_{KPC-33}$

The genetic environment of *bla*$_{KPC-102}$ was nearly identical to those of the *bla*$_{KPC-2}$ and *bla*$_{KPC-33}$, namely, *TnAs1-IS26-tnpR-ISKpn27-bla*$_{KPC}$*-hp-hp-klcA-hp-hp-hin-hp-IS26* for *bla*$_{KPC-2}$ and *bla*$_{KPC-33}$, *IS26-tnpR-ISKpn27-bla*$_{KPC}$*-hp-hp-klcA-hp-IS26* for *bla*$_{KPC-102}$ (Fig. 3B). In addition, MSA and LCB analysis revealed that partial nucleotide sequences of the adjacent genomes of *bla*$_{KPC}$ of Kp36854 were homologous to that of Kp37523. The sequences from position 35356 to 37269 and from position 45014 to 46813 on pKP36854-3 were homologous to the sequences ranging from position 2453 to 5983 on plasmid 2 of Kp37523 (pKP37523-2). Additionally, the sequences from positions 37320 to 43471 on pKP36854-3 were reverse-complementary homologous to the sequence ranging from position 25963 to 32114 of plasmid 4 of Kp38097 (pKP38097-4) (Fig. S2).

3.5. Characteristics of various KPC variants in three strains

The kinetic parameters of nitrocefin, meropenem, ceftazidime and avibactam were calculated as shown in Table 2. For nitrocefin, KPC-2 exhibited the highest catalytic efficiency (K_{cat}/K_m = 0.365 $\mu M^{-1}s^{-1}$), while KPC-33 showed a significant reduction (0.117 $\mu M^{-1}s^{-1}$) despite a lower K_m. KPC-δ33 retained some activity (0.250 $\mu M^{-1}s^{-1}$), but KPC-102 showed a marked decrease (0.0447 $\mu M^{-1}s^{-1}$). For meropenem, KPC-2 had the strongest efficiency (K_{cat}/K_m = 0.120 $\mu M^{-1}s^{-1}$), while KPC-δ33 and KPC-102 exhibited much lower values (0.101 and 0.0077 $\mu M^{-1}s^{-1}$). In ceftazidime hydrolysis, KPC-33 had a higher K_{cat}/K_m (0.0060 $\mu M^{-1}s^{-1}$), suggesting increased resistance, while other variants had limited activity. Regarding avibactam, KPC-2 showed the highest sensitivity (K_i = 0.558 μM), while KPC-δ33 and KPC-102 were less sensitive (K_i = 1.132 and 3.218 μM).

Meanwhile, the protein structure of KPC-102 was also modelled by using the template of KPC-2 (PDB ID: 4ZBE). As depicted in Fig. 4, the conformation of KPC-102 was different from that of

Table 1
Antibiotic susceptibility of *Klebsiella pneumoniae* strains, *E. coli* DH5α recipient and *bla*$_{KPC}$-positive *E. coli* DH5α transformants.

Strains[a]	Carbapenemase resistance genes	MIC (mg/L)[b]												
		IPM	MEM	CAZ	CZA	TZP	FEP	ATM	AK	TOB	CIP	LEV	PB	TGC
Kp36854	*bla*$_{KPC-2}$	64	256	2048	4/4	≥128	≥32	≥64	≤2	8	≥4	≥8	≤0.5	≤2
Kp37523	-	2	0.25	≥64	8/4	≥128	≥32	≥64	≥64	≥16	≥4	≥8	≥16	≥8
Kp38097	*bla*$_{KPC-102}$	1	1	1024	1024/4	≥128	≥32	≥64	≥64	≥16	≥4	≥8	≤0.5	≤2
Kp38935	*bla*$_{KPC-33}$	1	4	2048	512/4	≥128	≥32	≥64	≤2	8	≥4	≥8	≤0.5	≤2
E. coli DH5α	-	<0.064	<0.064	0.25	<0.032/4	≤4	≤1	≤1	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2
E. coli DH5α/pEasy-Blunt E2	-	<0.064	<0.064	0.25	<0.032/4	≤4	≤1	≤1	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2
E. coli DH5α/pKPC2	*bla*$_{KPC-2}$	32	32	128	0.125/4	32	16	≤1	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2
E. coli DH5α/pKPC33	*bla*$_{KPC-33}$	0.25	<0.064	1024	32/4	32	≥32	≤1	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2
E. coli DH5α/pKPC102	*bla*$_{KPC-102}$	0.125	<0.064	512	512/4	≥128	≥32	≤1	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2
E. coli DH5α/pKPCδ33	*bla*$_{KPC-\delta33}$	1	<0.25	128	32/4	32	≥32	2	≤2	≤1	≤0.25	≤0.25	≤0.5	≤2

[a] Kp36854, Kp37523, Kp38097 and Kp38935 were isolated from the patients in this study. *E. coli* DH5α/pEasy-Blunt E2 was transformed by expression plasmid pEasy-Blunt E2. *E. coli* DH5α/pKPC2 was transformed by pEasy-Blunt E2 plasmid carrying *bla*$_{KPC-2}$ gene from Kp36854. *E. coli* DH5α/pKPC33 was transformed by pEasy-Blunt E2 plasmid carrying *bla*$_{KPC-33}$ gene from Kp38935. *E. coli* DH5α/pKPC102 was transformed by pEasy-Blunt E2 plasmid carrying *bla*$_{KPC-102}$ gene from Kp38097. *E. coli* DH5α/pKPCδ33 was transformed by pEasy-Blunt E2 plasmid carrying *bla*$_{KPC-\delta33}$ gene. [b]IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; CZA, ceftazidime-avibactam; TZP, piperacillin-tazobactam; FEP, cefepime; ATM, aztreonam; AK, amikacin; TOB, Tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; PB, polymyxin B.

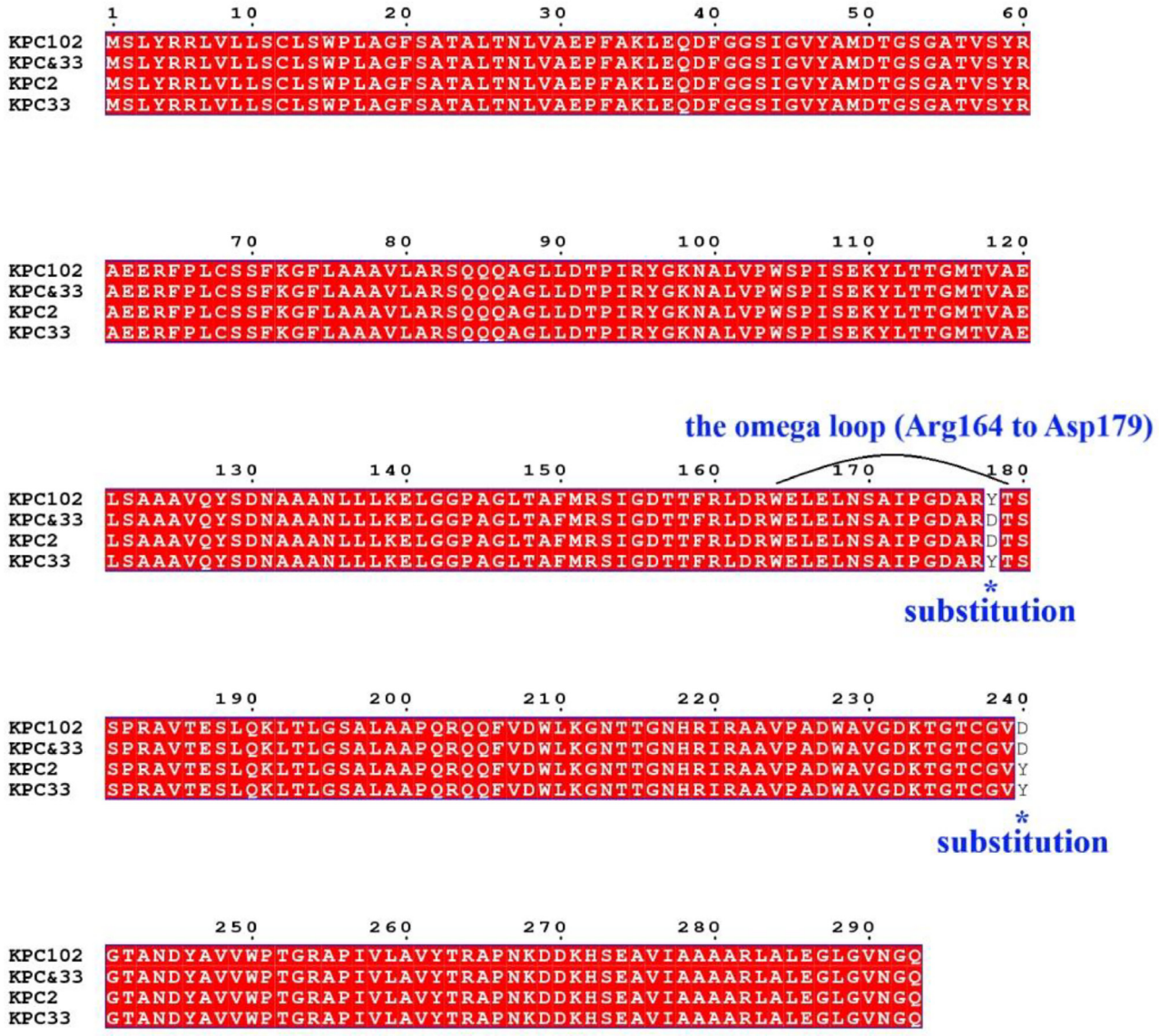

Fig. 2. Amplicon alignments between KPC-2, KPC-33, KPC-102 and KPC-δ33 in amino acid (aa) sequences surrounding the mutation. Mutated amino acids (Asp-Tyr) at amino acid sequence position 179 and (Tyr -Asp) at amino acid sequence position 241 were identified in the $bla_{KPC-102}$ gene when compared to bla_{KPC-2}.

KPC-2. Such conformational change of KPC-102 might be associated with the high-level resistance to CZA (Fig. 4).

NG-Test CARBA 5 showed negative results for Kp38097 ($bla_{KPC-102}$-positive) and Kp38935 (bla_{KPC-33}-positive) (Fig. S3A). While another immunochromatographic method (Goldstream Carbapenem-resistant K.N.I.V.O. Detection K-Set) showed positive results for Kp38097 ($bla_{KPC-102}$-positive) and Kp38935 (bla_{KPC-33}-positive) (Fig. S3B). Additionally, molecular screening of GeneXpert Carba-R showed good sensitivity for detecting bla_{KPC-2}, bla_{KPC-33}, and $bla_{KPC-102}$ (Fig. S3C).

To investigate the fitness effects of KPC variants, the growth curves of isolated strains and constructed strains were developed in the absence of antibiotics. Results showed that Kp38097 exhibited similar values of the maximum growth rate (V_{max}), the area under the growth curve (AUC) and the duration of the lag phase (DLP) compared to Kp36854 and Kp38935 (Fig. 5). In addition, similar values of V_{max} and AUC were observed in *E. coli* DH5α/pKPC2, *E. coli* DH5α/pKPC33, *E. coli* DH5α/pKPCδ33 and *E. coli* DH5α/pKPC102 strains, while the AUC value of the *E. coli* DH5α/pKPCδ33 strain was significantly lower than that of the *E. coli* DH5α/pKPC2, *E. coli* DH5α/pKPC33 and *E. coli* DH5α/pKPC102 strains. Therefore, antibiotic resistance emergence owing to the KPC-2, KPC-33 and KPC-102 could not influence the strain growth. Subsequently, the bla_{KPC-2}, bla_{KPC-33} and $bla_{KPC-102}$ genes were successfully transferred to Kp700603. The CZA MIC of Kp700603+pKPC2 was 0.5/4 mg/L, while the CZA MIC values of Kp700603+pKPC33 and Kp700603+pKPC102 were 32/4 mg/L and 128/4 mg/L, respectively.

4. Discussion

With the extensive usage of CZA, resistance mediated by inhibitor-resistant KPC variants rapidly emerged in KPC-Kp strains [12–15,24–26,28,29]. Currently, a variety of KPC variants capable of conferring CZA resistance have been identified, and the majority of these mutations occurred in the Ω-loop [24,30,31]. Of all, the D179Y mutation at the Ω-loop (amino acid positions 164-179) of the bla_{KPC-2} gene is the most common amino acid substitution associated with CZA resistance [19,29,32–35]. In addition, the amino acid substitution also occurred outside the Ω-loop region

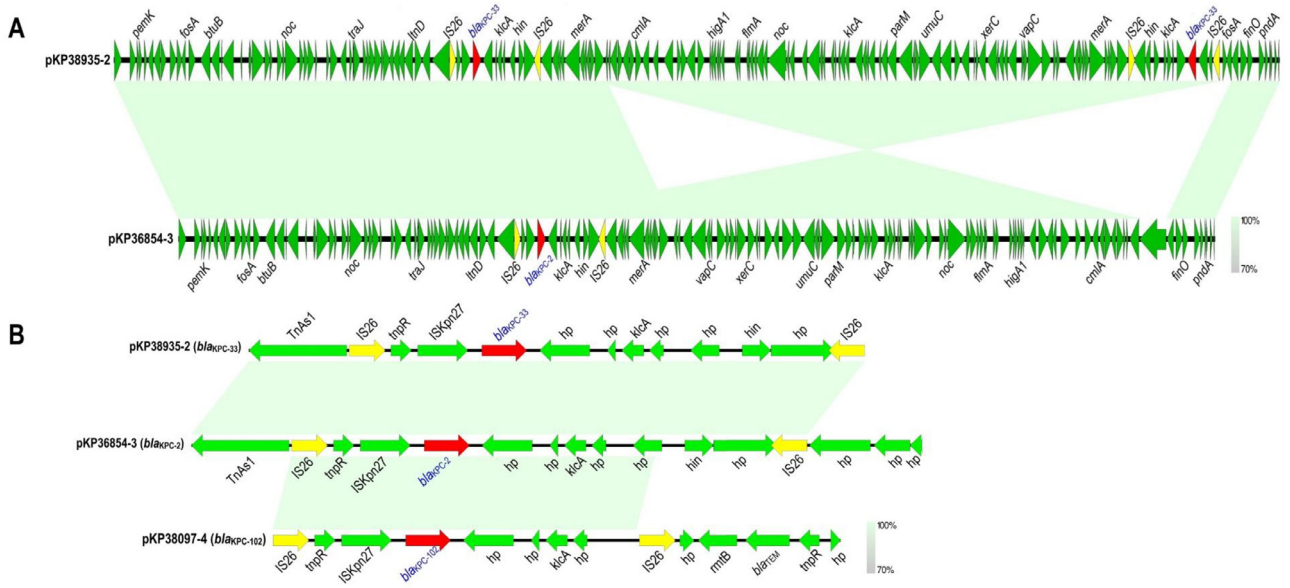

Fig. 3. Two plasmids pKP38935_2 and pKP36854_3, carrying bla_{KPC-33} and bla_{KPC-2} genes were depicted in Fig. 3A, respectively. The genetic environments of bla_{KPC-33}, bla_{KPC-2}, and $bla_{KPC-102}$ were described in Fig. 3B. Coding sequences are indicated by arrows. Sequences of shared homology between three plasmids are marked by the same shading.

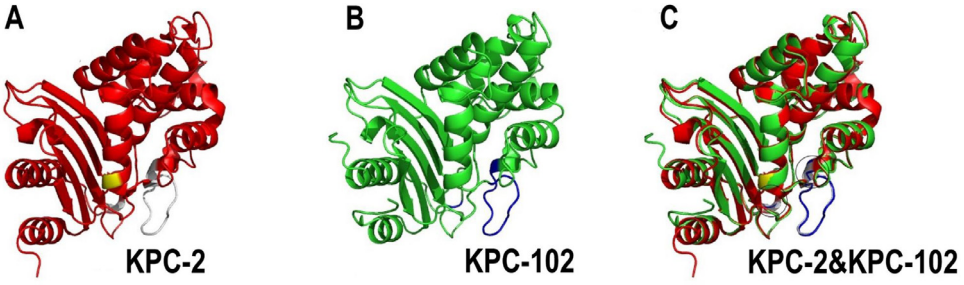

Fig. 4. Structure modeling of carbapenemase KPC-2 and KPC-102.

Table 2
Kinetic parameters of purified β-lactamases KPC variants.

		KPC-2	KPC-33	KPC-δ33	KPC-102
Nitrocefin					
	K_m (μM)	5.173	3.342	17.940	36.130
	K_{cat} (s^{-1})	1.886	0.3918	4.484	1.616
	K_{cat}/K_m (μM^{-1} s^{-1})	0.365	0.117	0.250	0.0447
Meropenem					
	K_m (μM)	11.10	ND[1]	15.53	25.13
	K_{cat} (s^{-1})	1.327	ND[1]	1.561	0.1938
	K_{cat}/K_m (μM^{-1} s^{-1})	0.120	ND[1]	0.101	0.0077
Ceftazidime					
	K_m (μM)	544.600	6.801	644.600	618.000
	K_{cat} (s^{-1})	0.0002784	0.00004086	0.0004452	0.0005104
	K_{cat}/K_m (μM^{-1} s^{-1})	0.0005	0.0060	0.0007	0.0008
Avibactam					
	K_i	0.558	ND[2]	1.132	3.218
	IC_{50} (μM)	9.182	ND[2]	6.180	10.343

Note: ND[1]: The value was not determined due to a low initial rate of hydrolysis. ND[2]: The value was beyond the detection line.

of the KPC-2 protein, such as KPC-74 (G239_V240del) and KPC-23 (Val240Ala and His274Tyr), which are also involved in the CZA resistance [10,36].

In this study, two CZA-susceptible strains (Kp36854 and Kp37523) and two CZA-resistant strains (Kp38097 and Kp38935) from one patient were analysed. Three KPC variants (KPC-2, KPC-33 and KPC-102) were identified in Kp36854, Kp38097 and Kp38935, different from the previous reports of KPC variants from the single Kp clone in one patient [11,12]. All three strains belonged to the ST11 clonal lineage. Notably, the novel KPC variant KPC-102 carried two amino acid mutations, including 179Asp-179Tyr within the Ω-loop, which is also harboured by KPC-33, and 241Tyr-241Asp outside the Ω-loop, which could synergistically contribute to high-level resistance to CZA [37,38].

The CZA MIC of *E. coli* DH5α/pKPC-102 carrying $bla_{KPC-102}$ was up to 512/4 mg/L while the CZA MIC of *E. coli* DH5α/pKPC-33 carrying bla_{KPC-33} was 32/4 mg/L, suggesting that amino acid mutation 241Tyr-241Asp outside the Ω-loop could also play a key role in CZA resistance. To verify this hypothesis, KPC-δ33 variant carrying only the amino acid mutation 241Tyr-241Asp was constructed. As expected, the CZA MIC of *E. coli* DH5α/pKPC-δ33 carrying $bla_{KPC-\delta33}$ was 32/4 mg/L (Table 1), indicating the association with amino acid mutation 241Tyr-241Asp outside the Ω-loop and CZA resistance. The results of the kinetic parameters of purified β-lactamases KPC variants could also explain the different CZA MIC values of different KPC variants.

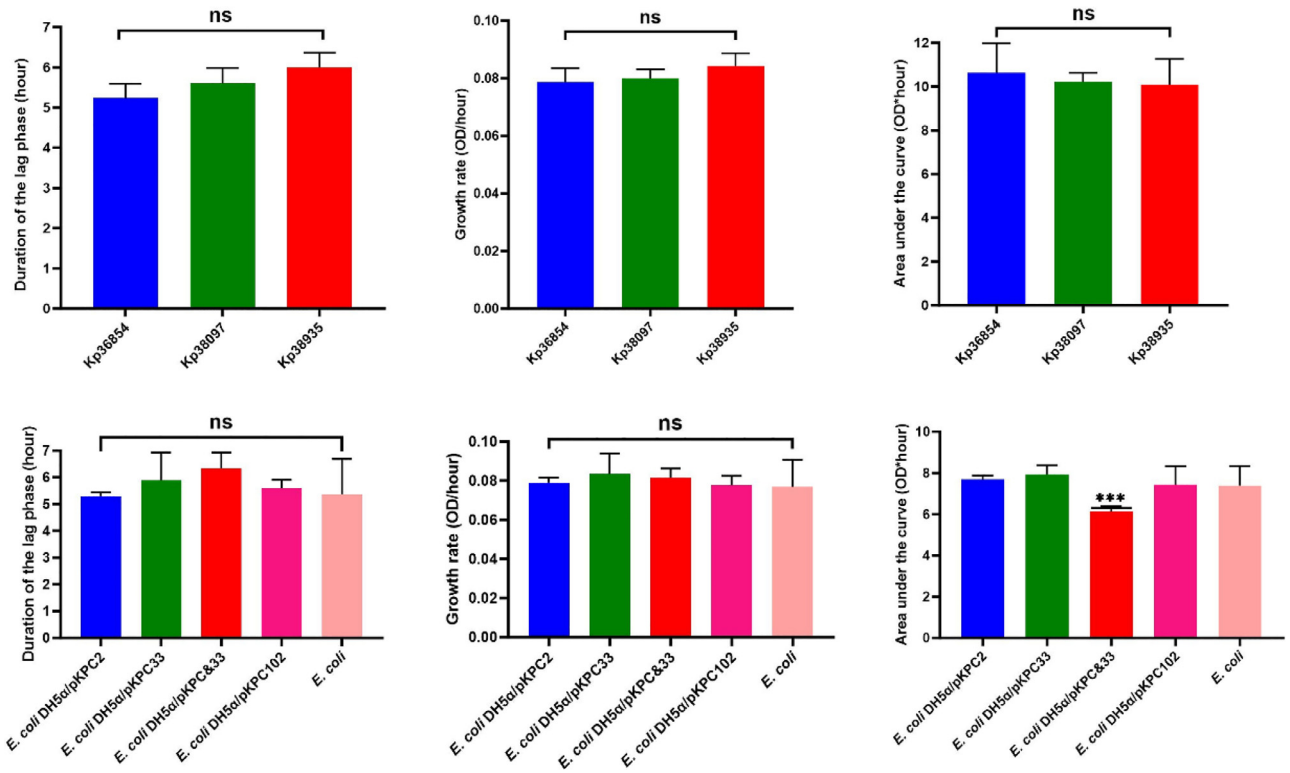

Fig. 5. The values of the maximum growth rate (V_{max}), the area under the growth curve (AUC) and the duration of the lag phase (lag) of isolated strains (Kp36854, Kp38097 and Kp38935) and constructed strains (*E. coli* DH5α, *E. coli* DH5α/pKPC2, *E. coli* DH5α/pKPC33 and *E. coli* DH5α/pKPC102).

It is of note that two copies of bla_{KPC-33} in Kp38935 were detected, and its expression level was found to be two-fold higher. In this study, we discovered that the CZA MIC of *E. coli* DH5α/pKPC-33 carrying bla_{KPC-33} was only 32/4 mg/L while the CZA MIC of Kp38935 strain is up to 512 mg/L, which could be the result of double copies of bla_{KPC-33} combined with Ompk35/36 defects. As for Kp38097, the mutated bla_{KPC} could be the most important mechanism of CZA resistance, and defects in porins might contribute limitedly to CZA resistance. Therefore, greater attention should be paid to CZA resistance resulting from bla_{KPC} gene mutations during continuous CZA exposure. However, identifying new KPC subtypes is difficult because of technical limitations in detection methods. With the rapidly rising occurrence of KPC variants, it is imperative to improve molecular screening methods to detect bla_{KPC} mutations and distinguish KPC variants more rapidly and accurately [39].

Recent studies revealed that the majority of CZA-resistant Kp strains were caused by multiple KPC mutations after treatment with CZA [13,21], as is confirmed by our study [10,12,15,24,28,29]. Unlike previous studies on KPC variants, these three Kp strains may not have originated from a single clone, as indicated by their distinct SNPs. Clearly, Kp38935 originated from Kp36854, but Kp38097 may have originated from other clones we failed to isolate. The results of MSA and LCB analysis of Kp36854, Kp37523, and Kp38097 genomes suggested that the partial nucleotide sequences from position 35356 to 37269 on pKP36854-3 could insert into the sequences from position 2453 to 4366 on pKP37523-2, while the sequences from position 37320 to 43471 on pKP36854-3 could insert reversely into the sequences from position 25963 to 32114 of pKP38097-4. The sequences from position 45014 to 46813 on pKP36854-3 might insert reversely into the sequences from position 4367 to 5983 on pKP37523-2. These findings need to be further verified. Additionally, the fact that the identical genetic context of pKP36854_3 carrying bla_{KPC-2} and pKP38935_2 carrying bla_{KPC-33} was suggestive of the further dissemination of KPC variants among bacteria evolved within the same host. Thus, it is urgently needed to take effective measures to prevent the spread of this resistance plasmid.

In conclusion, three KPC variants were identified from a lung transplantation recipient during the CZA treatment of CRKP infection. Notably, a novel KPC-102 variant with a mutation 179Asp-179Tyr within the Ω-loop and 241Tyr-241Asp outside the Ω-loop one and another KPC-33 variant with two copies of bla_{KPC-33} in porin-deficient Kp strains were found to confer high-level resistance to CZA after CZA exposure. This CZA resistance caused by bla_{KPC} gene mutation could be selected after CZA therapy and evolved to be more diverse and heterogeneous within the host. It is necessary to perform surveillance for CZA resistance during treatment to ensure adequate treatment of the patient and to prevent wider dissemination of CZA-resistant strains.

Data availability: Genome sequences for all involved isolates in this study have been registered under the BioProject number PRJNA1025953. The sequences of all isolates have been deposited under BioSample accession numbers SAMN37731363 (Kp36854), SAMN37731364 (Kp38097), SAMN37731365 (Kp38935) and SAMN40232522 (Kp37523), respectively.

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Declaration of competing interest: None declared.

Ethical approval: The Ethics Committee of the China-Japan Friendship Hospital (CJFH) approved this study (2022-KY-054).
Sequence information: I have deposited these contents in the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2025.107521.

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