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ORIGINAL RESEARCH

Characteristic of KPC-12, a KPC Variant Conferring Resistance to Ceftazidime-Avibactam in the Carbapenem-Resistant *Klebsiella pneumoniae* ST11-KL47 Clone Background

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Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections are a great threat to public health worldwide. Ceftazidime-avibactam (CZA) is an effective *β*-lactam/*β*-lactamase inhibitors against CRKP. However, reports of resistance to CZA, mainly caused by *Klebsiella pneumoniae* carbapenemase (KPC) variants, have increased in recent years. In this study, we aimed to describe the resistance characteristics of KPC-12, a novel KPC variant identified from a CZA resistant *K. pneumoniae*.

Methods: The *K. pneumoniae* YFKP-97 collected from a patient with respiratory tract infection was performed whole-genome sequencing (WGS) on the Illumina NovaSeq 6000 platform. Genomic characteristics were analyzed using bioinformatics methods. Antimicrobial susceptibility testing was conducted by the broth microdilution method. Induction of resistant strain was carried out in vitro as previously described. The *G. mellonella* killing assay was used to evaluate the pathogenicity of strains, and the conjugation experiment was performed to evaluate plasmid transfer ability.

Results: Strain YFKP-97 was a multidrug-resistant clinical ST11-KL47 *K. pneumoniae* confers high-level resistance to CZA (16/4 µg/mL). WGS revealed that a KPC variant, KPC-12, was carried by the IncFII (pHN7A8) plasmids (pYFKP-97_a and pYFKP-97_b) and showed significantly decreased activity against carbapenems. In addition, there was a dose-dependent effect of *bla*$_{\text{KPC-12}}$ on its activity against ceftazidime. In vitro inducible resistance assay results demonstrated that the KPC-12 variant was more likely to confer resistance to CZA than the KPC-2 and KPC-3 variants.

Discussion: Our study revealed that patients who was not treated with CZA are also possible to be infected with CZA-resistant strains harbored a novel KPC variant. Given that the transformant carrying *bla*$_{\text{KPC-12}}$ was more likely to exhibit a CZA-resistance phenotype. Therefore, it is important to accurately identify the KPC variants as early as possible.

Keywords: carbapenem-resistant *Enterobacterales*, *Klebsiella pneumoniae*, ceftazidime-avibactam, KPC-12, carbapenemase

Introduction

The global dissemination of carbapenem-resistant *Enterobacterales* (CRE) has emerged as an urgent public health concern due to extremely limited antibiotic treatment options.[1–3] Recognizing the significant threats posed by CRE, the World Health Organization has classified it as a priority antimicrobial resistant pathogen. It is worth mentioning that carbapenem-resistant *Klebsiella pneumoniae* strains (CRKP) account for over half of the clinical CRE infections in the United States, Europe and China, resulting in increased mortality rates.[4–6]

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Klebsiella pneumoniae, famous for its tendency to acquire multidrug resistance plasmids, is a notorious producer of *Klebsiella pneumoniae* carbapenemase (KPC)-type class A carbapenemases. The first KPC-positive *K. pneumoniae* strain was collected from the United States in the 1990s.[7] Shortly afterwards, the first KPC-producing *K. pneumoniae* strain reported in China was isolated from an ICU patient in Zhejiang Province in 2007.[8] With the increasing clinical use of carbapenems in recent decades, the prevalence of CRKP has also increased at an alarming rate. In China, approximately 60% of the CRKP strains produce KPC, with KPC-2 being the most prevalent carbapenemase.[9] Whereas, in the United States, Italy, and Israel KPC-3 is the predominant KPC-type carbapenemases.[10] What's more, it has been showed that mortality rates of patients infected with CRKP are much higher than those who infected with carbapenem-susceptible *K. pneumoniae* (CSKP).[11] Therefore, there is an urgent need to address the treatment challenges associated with CRKP.

Given the potent ability of KPC to hydrolyze almost all carbapenems, a novel combination of a *β*-lactam antibiotic and *β*-lactamase inhibitor, ceftazidime-avibactam, was approved for clinical use by the Food and Drug Administration (FDA) in 2015 and was implemented in China in 2019. However, according to information from the National Center for Biotechnology Information (NCBI) Reference Sequence database (Reference Gene Catalog - Pathogen Detection - NCBI (nih.gov)), there has been an upsurge in reports of resistance to ceftazidime-avibactam (CZA) due to mutations derived from the $bla_{\text{KPC-2}}$ or $bla_{\text{KPC-3}}$ genes, which can be mainly attributed to the widespread use of CZA in clinical practice.

The purpose of this study was to characterize a CRKP isolate from a patient with respiratory infection by WGS. A novel KPC variant, KPC-12, was identified, and in vitro assays were performed to reveal its resistance properties. Additionally, we identified the threats posed by $bla_{\text{KPC-12}}$ carriers in the development of CZA-resistant phenotypes as CZA explored. Overall, this study aimed to elucidate the features of this KPC variant and emphasize the potential treatment dilemma for CRKP strains.

Methods and Materials

Bacterial Strains and Antimicrobial Susceptibility Test

We collected 187 unique CRE isolates from patients with respiratory tract infections between July 2019 and June 2021 at a teaching hospital in Shanghai, China. Among these, *K. pneumoniae* YFKP-97 was selected for further investigation to determine its resistance to CZA. The MIC of meropenem, imipenem, ertapenem, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, cefepime, cefotaxime, ceftazidime-avibactam, cefoperazone/sulbactam, piperacillin-tazobactam, amoxicillin/clavulanic acid, amikacin, levofloxacin, tigecycline, and trimethoprim/sulfamethoxazole were determined using the broth microdilution method following the Clinical and Laboratory Standards Institute guidelines. *E. coil* ATCC25922 was used as quality control for the antimicrobial susceptibility test (AST). *K. pneumoniae* HS11286 (harboring $bla_{\text{KPC-2}}$[12]) and FK3015 (harboring $bla_{\text{KPC-3}}$[13]) strains were used as reference to compare antimicrobial resistance and virulence pattern of *K. pneumoniae* YFKP-97 strain. The MICs were interpreted according CLSI breakpoint,[14] except tigecycline, which was interpreted based on the Food and Drug Administration (FDA) guidelines.

Whole-Genome Sequencing and Bioinformatics Analysis

Genomic DNA of *K. pneumoniae* YFKP-97 was extracted using a commercial genomic DNA extraction kit (Qiagen, Germany), according to the manufacturer's instructions, and genome sequencing was performed using the Illumina NovaSeq 6000 platform with 2 × 125-bp paired-end libraries. Long-read sequencing was also performed using the PacBio Sequel platform with a 10-kb library. After removing adapter contamination, data assembly was performed and data filtering was performed using AdapterRemoval[15] and SOAPec.[16] Filter reads were assembled into scaffolds and contigs using SPAdes (version 3.12)[17] and A5-miseq (version 20160825).[18] The CANU (version 1.7.1)[19] software was used to assemble the data acquired by PacBio platform sequencing. Subsequently, the complete sequence was generated by integrating the entire assembled sequence, and the genome sequence was corrected using Pilon software. The assembled genome sequences were annotated using RAST (version 2.0).[20] The ST of the isolates and serotypes were determined using MLST (MLST 2.0 (dtu.dk)) and kleborate (GitHub - klebgenomics/Kleborate). The acquired antibiotic resistance genes were identified using ResFinder (Center for Genomic Epidemiology (dtu.dk)) with the default threshold. ISfinder (biotoul.fr) was used to determine the related insertion sequences (ISs) and transposons (Tns). Orifinder (sjtu.

edu.cn) was used to predict whether plasmids could be self-transmitted. Proksee (Proksee - Genome Analysis) software was used to compare pYFKP-97_a and pYFKP-97_b with other representative plasmids to generate circular plasmid maps. A comparison of the gene environments surrounding the bla_{KPC-12} within pYFKP-97_a and pYFKP-97_b plasmids was performed using Easyfig software. Sequences were aligned using the ClustalW sequence alignment tool in MEGA 11.0. Alignment of the amino acid sequences and prediction of the secondary structures of KPC-2, KPC-3, and KPC-12 were performed using ESPript 3.0.[21]

KPC-Plasmid Transformation

Purified plasmid DNA was obtained from overnight Luria-Bertani (LB) liquid broth cultures of the selected strains. The bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-12} genes, which contained the promoter region, were amplified using the primers listed in Table 1. The PCR products were recombined into the vector pACYC184, which was further transformed into *Escherichia coli* strain DH5α via chemical transformation experiments. Transformants were selected on LB agar plates containing 10 μg/mL chloramphenicol and verified by bla_{KPC} gene PCR and Sanger sequencing.

Induction of Resistant Strain

Induction of strains to the CZA-resistance phenotype was carried out according to a previously described method.[22] Single randomly selected colonies of DH5α::pACYC184-bla_{KPC-2}, DH5α::pACYC184-bla_{KPC-3} and DH5α::pACYC184-bla_{KPC-12} were incubated overnight, and then freshly inoculated bacterial broth was diluted in 4 mL LB medium containing 1/8 × the MIC of CZA and incubated overnight at 37 °C with 220 rpm (Figure 1B). After five passengers, aliquots were collected to determine MIC. In the following five generations, LB medium containing 1/4 μg/mL, 4/4 μg/mL and 8/4 μg/mL CZA was used to culture strains carrying bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-12}, respectively (Figure 1B). The activity of CZA against ten generations was also evaluated using the broth microdilution method.

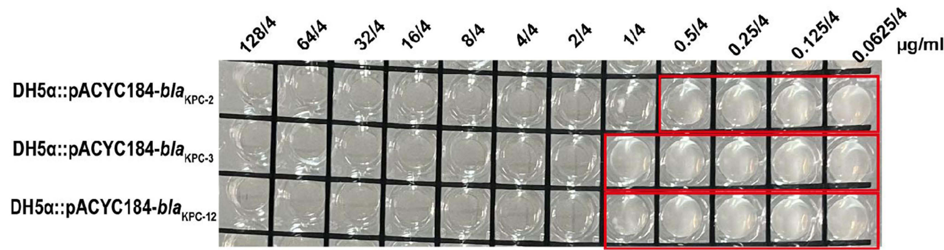
Conjugation Assay

We used a conjugation assay to evaluate whether resistant plasmids harboring bla_{KPC-12} could be transferred from *K. pneumoniae* YFKP-97 (donor isolate) to *E. coli* EC600 (recipient isolate). The donors and recipients were cultured in LB broth to the logarithmic phase at 37 °C with a mix ratio of 1:1, and then centrifuged at 8000 × g for 1 min, and resuspended in 20 μL Luria-Bertani. The suspension was spotted on an LB plate and incubated at 37 °C overnight. Subsequently, serial dilutions were plated in medium containing specific antibiotics (meropenem, 2 μg/mL [bla_{KPC-12}]; rifampicin, 200 μg/mL [EC600 recipient]).

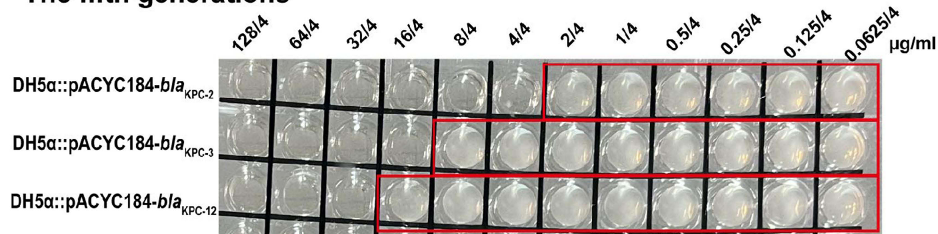
Table 1 Oligonucleotides for PCR

Name	Sequence (5'-3')
bla_{KPC-2}-pro-F	GCCCTTAAACGCCTGGTGCTACGCCTGAATAAGTTCAAATATGTATCCGC
bla_{KPC-2}-pro-R	ATCACAGTTAAATTGCTAACGCAGTCAGGCTTACTGCCCGTTGACGCCCA
bla_{KPC-3}-pro-F	CTTAAACGCCTGGTGCTACGCCTGAATAAGCATGGGGGATCATGTAACTC
bla_{KPC-3}-pro-R	ATCACAGTTAAATTGCTAACGCAGTCAGGCTTACTGCCCGTTGACGCCCA
bla_{KPC-12}-pro-F	GCCCTTAAACGCCTGGTGCTACGCCTGAATAAGTTCAAATATGTATCCGC
bla_{KPC-12}-pro-R	ATCACAGTTAAATTGCTAACGCAGTCAGGCTTACTGCCCGTTGACGCCCA
pACYC184-F	CTTATTCAGGCGTAGCACCA
pACYC184-R	GCCTGACTGCGTTAGCAATT
bla_{KPC-12}-double-F	CGGCTAGCCGCATGAGCGGATACATATTTGAA
bla_{KPC-12}-double-R	CGGCTAGCCGTTACTGCCCGTTGACGCCCAAT
q- bla_{KPC-12}-F	CCGTCATGCCTGTTGTCAGA
q-bla_{KPC-12}-R	TGGACACACCCATCCGTTAC
q-*rrsA*-F	GACTTGGAGGTTGTGCCCTT
q-*rrsA*-R	TTTAACCTTGCGGCCGTACT

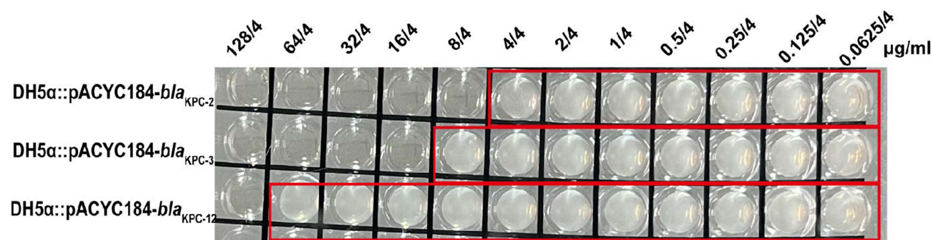
(A) The primary generations

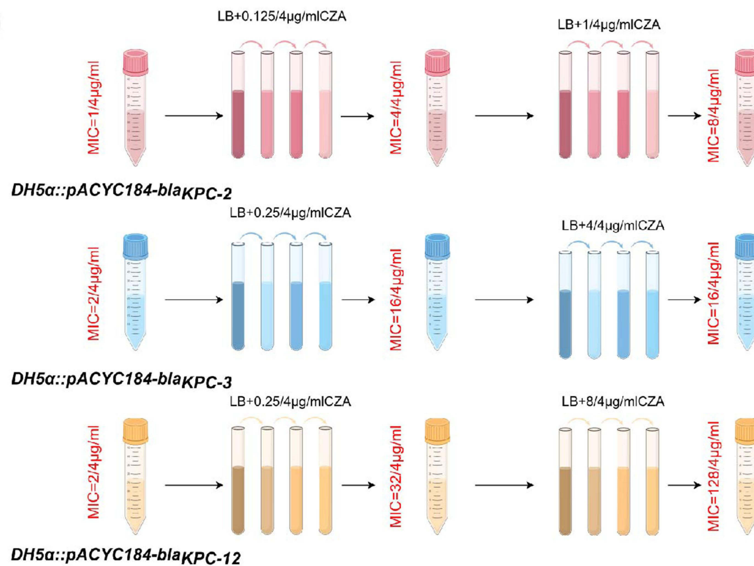


The fifth generations



The tenth generations





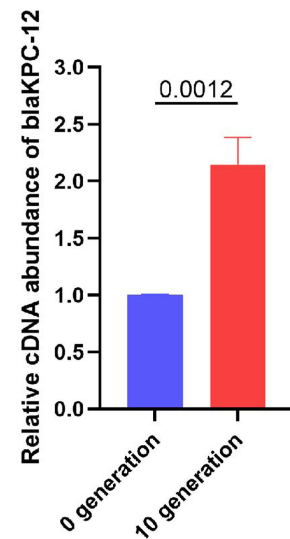


Figure 1 The CZA susceptibility test results for strains carrying bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-12}, as well as their corresponding inducible resistant strains. (**A**) The MIC values were determined by the broth microdilution method. The box in red indicates that the bacteria were able to grow at those concentrations of antibiotics. (**B**) The schematic diagram illustrating the process of CZA resistance induction was draw by Figdraw (ID: TSIPYb866b). The MIC of CZA for the respective strains were displayed in red text in a format of "MIC=X/X μg/mL". (**C**) The relative expression levels of bla_{KPC-12} in DH5α::pACYC184-bla_{KPC-12} before and after 10 generations. All results are presented as the means ± SDs. Statistically significant difference was determined by the Student's *t*-test and *P* values less than 0.05 were considered statistically significant.

G. mellonella Killing Assays

We used a *G. mellonella* killing assay to evaluate the pathogenicity of *K. pneumoniae* YFKP-97, NUTH-K2044 and HS11286. *G. mellonella* caterpillars were stored at 4 °C before use, after regaining activity at 37 °C for 2 hours, each group of the caterpillars were inoculated with 10 μL of at a concentration of 1.0×10^7 CFU/mL *K. pneumoniae* YFKP-97, NUTH-K2044 and HS11286. The saline-treated group was used as the control. The caterpillars were placed in Petri dishes at 37 °C, and the survival rates were recorded every 8 h.

Total RNA Extraction, cDNA Synthesis and Real-Time PCR

Bacteria were grown to mid-exponential phase at 37 °C in LB medium. After centrifugation at 12,000 × g for 2 min, cells were resuspended in TE buffer containing 20mg/mL lysozyme and incubated at 37 °C for 20 min. After digestion, total RNA was extracted according to the manufacturer's instructions [Spin Column Bacteria Total RNA Purification Kit; Sangon Biotech Co. Ltd., Shanghai, China]. RNA was reverse transcribed into cDNA using a cDNA synthesis kit (Takara, Tokyo, Japan). Quantitative Real-Time PCR was performed using TB Green Master Mix (Takara, Tokyo, Japan). The primers used for RT-qPCR are listed in Table 1. Relative expression values were calculated using the $2^{-\Delta\Delta CT}$ method with the stable housekeeping gene *rrsA* as the normalizer.

Results

Overview of the Clinical History

The CRKP isolate, designated YFKP-97, was collected from the sputum specimen of an 80-year-old man admitted to Shanghai Pulmonary Hospital, Tongji University, in 2020. The patient was hospitalized for several weeks because of a tuberculosis infection. After administration of empirical anti-tuberculosis treatment (ethambutol, levofloxacin, and linezolid) for approximately 12 days, the patient experienced a critical respiratory infection accompanied by the detection of *K. pneumoniae* YFKP-97. To characterize the antibiotic-resistant phenotype of *K. pneumoniae* YFKP-97, we tested its susceptibility to 17 antibiotics. As shown in Table 2, *K. pneumoniae* YFKP-97 was a typical multidrug resistant strain that was resistant to cephalosporins, carbapenems, *β*-lactam/*β*-lactamase inhibitors, amikacin, and levofloxacin but susceptible to tigecycline (MIC=2 μg/mL).

Phenotypic Characteristics of K. pneumoniae YFKP-97

We compared the virulence of *K. pneumoniae* YFKP-97 with NUTH-K2044 (a strain of *K. pneumoniae* exhibiting high virulence) and HS11286 (a strain of *K. pneumoniae* exhibiting low virulence[12]) using a *Galleria mellonella*-infection model. As illustrated in Figure 2, *K. pneumoniae* YFKP-97 appeared to be slightly more virulent than *K. pneumoniae* HS11286. The 48-hour survival rate of *Galleria mellonella* infected with *K. pneumoniae* YFKP-97 was 30%, which was lower than that in the group infected with *K. pneumoniae* HS11286 (60%), but higher than that of the group infected with NUTH-K2044 (0%). A significant difference ($P < 0.0001$) was observed between the groups. To further clarify the carbapenem-resistance phenotypes of CRKP YFKP-97, we compared the resistance profiles of *K. pneumoniae* YFKP-97 with those of two dominant bla_{KPC} carriers, HS11286 (harboring bla_{KPC-2}[12]) and FK3015 (harboring bla_{KPC}-3)[13] We found that *K. pneumoniae* YFKP-97 posed comparable multidrug-resistant features to those of HS11286 and FK3015; they exhibited high-level resistance to all *β*-lactam antibiotics (Table 2). Notably, *K. pneumoniae* YFKP-97 showed a higher level of resistance to CZA than HS11286 (KPC-2) or FK3015 (KPC-3). The MIC of CZA for YFKP-97 was 16/4 μg/mL, which was four times higher than that for HS11286 (4/4 μg/mL) and FK3015 ((4/4 μg/mL)). Afterwards, we performed WGS to further explore the genetic features of *K. pneumoniae* YFKP-97, which harbors these interesting resistance phenotypes.

Genomic Characteristics of K. pneumoniae YFKP-97

According to WGS analysis, YFKP-97 has a 5,384,124-bp chromosome and two plasmids: pYFKP-97_a and pYFKP-97_b. Multi-locus sequence typing showed that YFKP-97 belongs to ST11 *K. pneumoniae*, the predominant KPC-producing *K. pneumoniae* (KPC-KP) sequence type (ST) in Asia and Latin America.[2] Isolate YFKP-97 belongs to the KL47 capsule

Table 2 Antimicrobial Drug Susceptibility Profiles of Associated *K. pneumoniae* in This Study

Antibiotics	MIC(μg/mL)		
	YFKP-97	HS11286	FK3015
MEM	64(R)	16(R)	32(R)
IPM	32(R)	≥16(R)	≥16(R)
ETP	≥8(R)	≥8(R)	≥8(R)
FOX	≥64(R)	16(R)	≥64(R)
CAZ	≥64(R)	32 (R)	≥64(R)
CRO	≥64(R)	≥64(R)	≥64(R)
CXM	≥64(R)	≥64(R)	≥64(R)
FEP	≥32(R)	≥32(R)	≥8(R)
CTX	1024(R)	128(R)	1024(R)
CZA	16/4(R)	4/4(S)	4/4(S)
CSL	≥64/128(R)	≥64/128(R)	≥64/128(R)
TZP	≥128/4(R)	≥128/4(R)	≥128/4(R)
AMC	≥32(R)	≥32(R)	≥32(R)
AMK	≥64(R)	≥64(R)	≤2 (S)
LVX	≥8(R)	4(R)	≤0.12 (S)
TGC	2(S)	1(S)	1(S)
SXT	≤20 (S)	≥320(R)	≤20 (S)

Abbreviations: MIC, minimal inhibitory concentration; MEM, meropenem; IPM, imipenem; ETP, ertapenem; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; CXM, cefuroxime; FEP, cefepime; CTX, cefotaxime; CZA, ceftazidime-avibactam; CSL, cefoperazone/sulbactam; TZP, piperacillin-tazobactam; AMC, amoxicillin/clavulanic acid; AMK, amikacin; LVX, levofloxacin; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole.

type and lacks several virulence genes associated with siderophore biosynthesis, including *iucA, iucB, iucC, iucD, iroB, iroC, iroD* and *rmpA*.

Genomic Characteristics of K. pneumoniae YFKP-97 Carried Plasmids

ResFinder analysis of the plasmids revealed that both pYFKP-97_a and pYFKP-97_b harbored *bla*$_{KPC-12}$, a variant of KPC-2. Nucleotide alignment of *bla*$_{KPC-2}$ and *bla*$_{KPC-12}$ showed that *bla*$_{KPC-12}$ differs from *bla*$_{KPC-2}$ by a C>A substitution at position 502 (Figure 3A), resulting in an L169M substitution in the Ω loop (Figure 3B). pYFKP-97_a was

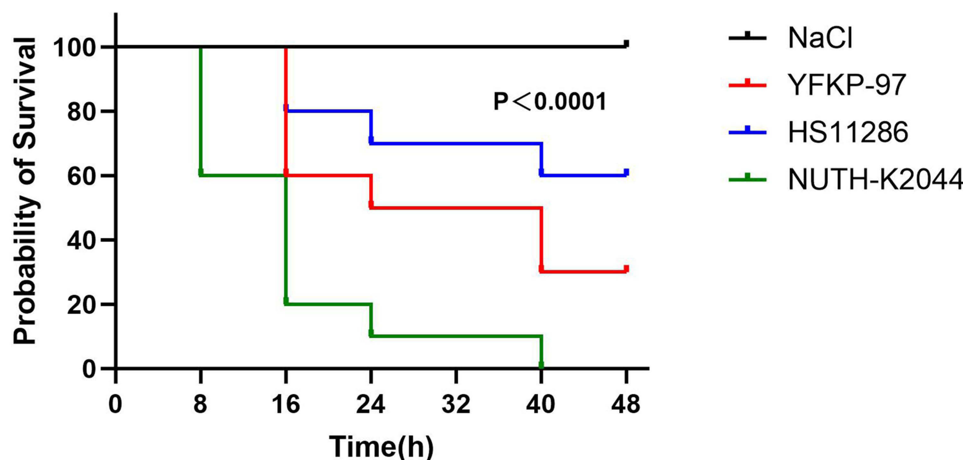


Figure 2 Survival rates of *Galleria mellonella* infected with *K. pneumoniae* YFKP-97, HS11286 and NUTH-K2044. A log-rank (Mantel-Cox) test was performed for the indicated curves. A significant difference (*P*<0.0001) was observed between these groups.
Abbreviation: NaCl, normal saline.

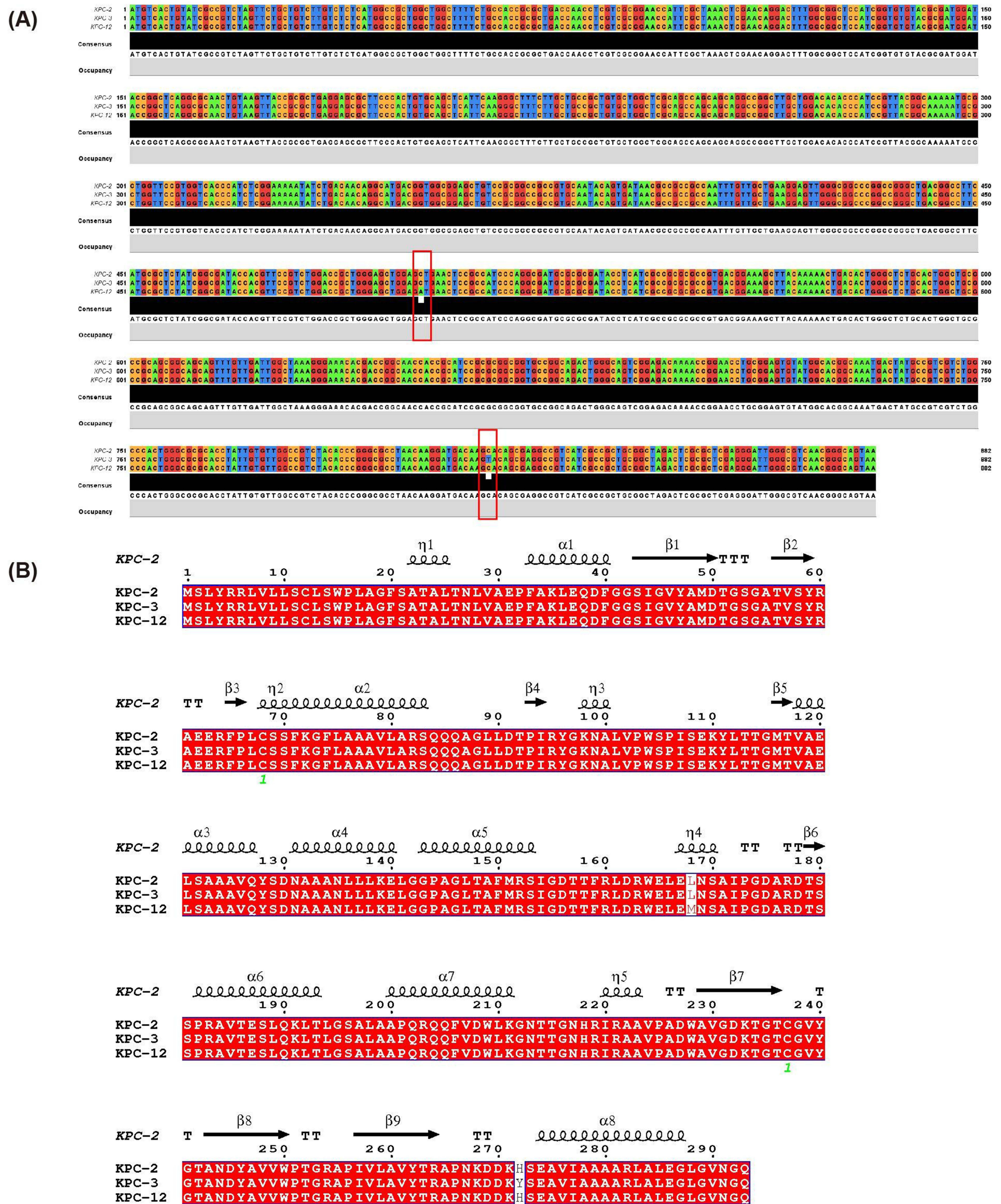


Figure 3 (**A**) The nucleotide sequence alignments of *bla*$_{KPC-2}$, *bla*$_{KPC-3}$ and *bla*$_{KPC-12}$. The differences in the nucleotide sequence of *bla*$_{KPC-2}$, *bla*$_{KPC-3}$ and *bla*$_{KPC-12}$ were highlighted by using red frames. (**B**) The alignments of amino acid sequences and the predicted secondary structures of KPC-2, KPC-3 and KPC-12.

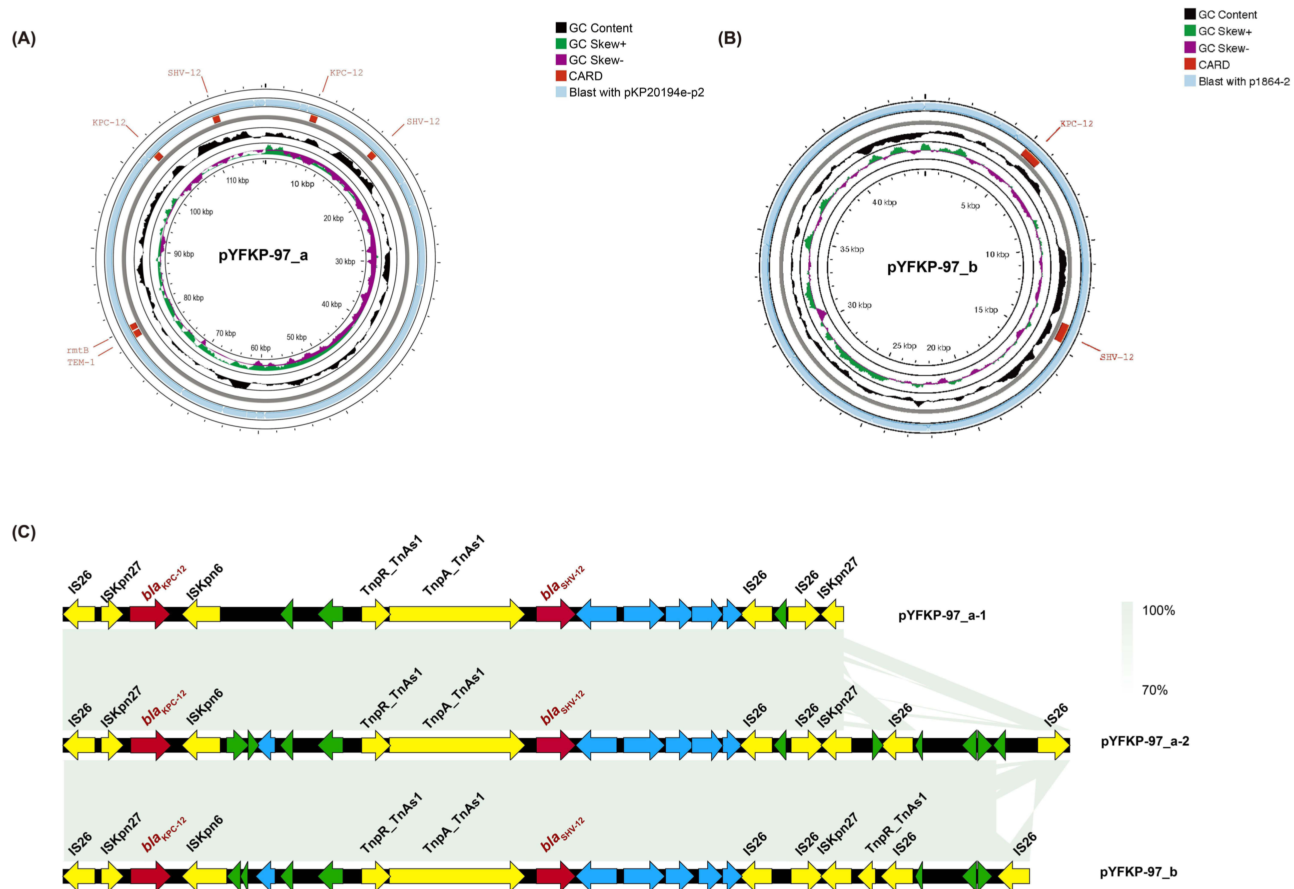


Figure 4 Comparative analysis of pYFKP-97_a and pYFKP-97_b plasmids with other reference plasmids. (**A**) Schematic map of plasmid pYFKP-97_a. The sequence alignment between pYFKP-97_a and pKP20194e-p2 (GenBank accession no. CP054728) is shown in the circle in blue. (**B**) Schematic map of plasmid pYFKP-97_b. The sequence alignment between pYFKP-97_b and p1864-2 (GenBank accession no. CP084494) is shown in the circle in blue. (**C**) Alignment of the genetic environment surrounding *bla*$_{KPC-12}$ within the pYFKP-97_a and pYFKP-97_b plasmids. The antimicrobial resistance genes are shown in red, mobile genetic elements are shown in yellow, open reading frames (ORFs) with specific functions are shown in blue and unidentified ORFs are shown in green. Green shading indicates regions of shared homology among different elements.

a 118,431-bp IncFII (pHN7A8) plasmid carrying two copies of *bla*$_{KPC-12}$, as well as the additional resistance genes *bla*$_{SHV-12}$, *bla*$_{TEM-1}$ and *rmtB* (Figure 4A). pYFKP-97_a had a GC content of 53.96%, 98% query coverage, and 99.99% identity with plasmid pKP20194e-p2 (GenBank accession no. CP054728) isolated from carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) in China.[23] Nevertheless, only one copy of *bla*$_{KPC-12}$ was located in pYFKP-97_b, a 44,545-bp IncFII plasmid with a GC content of 52.64% (Figure 4B). The antimicrobial resistance gene *bla*$_{SHV-12}$ was also detected in pYFKP-97_b. Further sequence alignment revealed that the plasmid nucleotide sequence of pYFKP-97_b was identical (100% coverage and 99.97% identity) to that of p1864-2 (GenBank accession no. CP084494), which harbored *bla*$_{KPC-2}$ but lacked a complete conjugation module and was carried by multidrug-resistant (MDR) *K. pneumoniae* 1864.[24] The genetic structures of *bla*$_{KPC-12}$ in pYFKP-97_a and pYFKP-97_b were identical and presented a typical Tn6296 flanking structure composed of two insertion sequences, IS*Kpn27* and IS*Kpn6*, a transposase gene (*tnpA*), a resolvase gene (*tnpR*), and *bla*$_{KPC}$ (Figure 4C).

We did not observe a complete conjugation module in either pYFKP-97_a or pYFKP-97_b, which lacked three essential conjugation components: *oriT*, relaxase, and T4CP protein. To verify the inability of the *bla*$_{KPC-12}$-harbouring plasmids obtained in this study to transfer, we applied a conjugation assay to evaluate whether these two plasmids carrying *bla*$_{KPC-12}$ could be transferred from *K. pneumoniae* YFKP-97 (donor isolate) to *E. coil* EC600 (recipient isolate). However, as previously assumed, we were unable to obtain transconjugants despite repeated matings. This finding illustrated that *bla*$_{KPC-12}$ carrying plasmids pYFKP-97_a and pYFKP-97_b could not self-transfer horizontally.

KPC-12 Exhibits Decreased Carbapenem Activity Compared to KPC-2 and KPC-3

To investigate the phenotypic properties of KPC-12, a 1062-bp amplicon containing the entire coding sequence of $bla_{\mathrm{KPC\text{-}12}}$ and the predicted upstream promoter was cloned into the pACYC184 vector and transformed into *E. coli* strain DH5α. Constructs containing $bla_{\mathrm{KPC\text{-}2}}$ and $bla_{\mathrm{KPC\text{-}3}}$ were used for comparison. The clones carrying $bla_{\mathrm{KPC\text{-}2}}$ and $bla_{\mathrm{KPC\text{-}3}}$ in the pACYC-184 vector exhibited higher resistance to multiple *β*-lactams than those carrying $bla_{\mathrm{KPC\text{-}12}}$, including meropenem (2 μg/mL for KPC-2, 4 μg/mL for KPC-3 and 0.06 μg/mL for KPC-12), imipenem (16 μg/mL for KPC-2, 16 μg/mL for KPC-3 and 1 μg/mL for KPC-12), cefuroxime (1024 μg/mL for KPC-2, ≥1024 μg/mL for KPC-3 and 256 μg/mL for KPC-12), cefepime (2 μg/mL for KPC-2, 2 μg/mL for KPC-3 and 1 μg/mL for KPC-12), and cefotaxime (16 μg/mL for KPC-2, 32 μg/mL for KPC-3 and 4 μg/mL for KPC-12) (Table 3). In addition, the L169M substitution in the Ω loop only resulted in a 1-fold increase in ceftazidime-avibactam MIC compared to that of KPC-2, indicating that it does not significantly affect susceptibility to ceftazidime-avibactam as previously believed. Taken together, KPC-12 was responsible for restoring susceptibility to carbapenems compared to $bla_{\mathrm{KPC\text{-}2}}$.

We also found that the activity of KPC-12 against cefoxitin was stronger than that of KPC-2 and KPC-3. This finding indicates that the misuse of cefoxitin, which has long been recognized as an effective cephamycin against certain multidrug-resistant *K. pneumoniae* strains,[25] may add to the treatment burden for patients infected with CRKP-producing KPC-12.

Dose-Dependent Impact of $bla_{\mathrm{KPC\text{-}12}}$ Gene on the Activity Against Ceftazidime

Based on the presence of two different plasmids carrying different copy numbers of $bla_{\mathrm{KPC\text{-}12}}$ in YFKP-97, we further constructed a single plasmid carrying two copies of $bla_{\mathrm{KPC\text{-}12}}$ to investigate whether a dosage effect exists for $bla_{\mathrm{KPC\text{-}12}}$ on its activity against carbapenems, similar to what has been reported for other carbapenemase genes.[26–28] As shown in Table 3, the MIC of ceftazidime for DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}12}}$*2, which has the simultaneous presence of two copies of $bla_{\mathrm{KPC\text{-}12}}$ in DH5α background, increases 4-fold compared to those for DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}12}}$. This result verified that the presence of double copies of $bla_{\mathrm{KPC\text{-}12}}$ was significantly correlated with increased resistance to ceftazidime, indicating the potential risk of decreased efficacy of ceftazidime-based therapies.

Table 3 Antimicrobial Drug Susceptibility Profiles of *E. coli* DH5α Transformants

Antibiotics	MIC(μg/mL)					
	DH5α::pACYC184	DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}12}}$	DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}12}}$*2	DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}2}}$	DH5α::pACYC184-$bla_{\mathrm{KPC\text{-}3}}$	ATCC25922
MEM	0.06(S)	0.06(S)	0.06(S)	2(S)	4(S)	0.03(S)
IPM	0.25(S)	1(S)	1(S)	16(R)	16(R)	≤0.25(S)
ETP	≤0.12(S)	0.25(S)	0.25(S)	0.25(S)	0.5(S)	≤0.12(S)
FOX	≤4(S)	32(R)	≥64(R)	16(I)	16(I)	≤4(S)
CAZ	0.25(S)	4(S)	16(I)	4(S)	128(R)	0.5(S)
CRO	≤0.25(S)	≥64(R)	≥64(R)	≥64(R)	≥64(R)	≤0.25(S)
CXM	8(S)	256 (R)	256 (R)	1024(R)	≥1024(R)	4(S)
FEP	≤0.12(S)	1(S)	1(S)	2(S)	2(S)	≤0.12(S)
CTX	1(S)	4(S)	4(S)	16(I)	32(I)	2(S)
CZA	1/4(S)	2/4(S)	2/4(S)	1/4(S)	2/4(S)	0.5/4(S)
CSL	≤8/128(S)	≤8/128(S)	16/128(S)	16/128(S)	32/128(R)	≤8/128(S)
TZP	≤4/4(S)	32/4(I)	64/4(I)	≥128/4(R)	≥128/4(R)	≤4/4(S)
AMC	≤2(S)	≥32(R)	≥32 (R)	≥32(R)	≥32(R)	4(S)
AMK	8(S)	4(S)	4(S)	4(S)	4(S)	4(S)
LVX	0.015(S)	0.03(S)	0.03(S)	0.03 (S)	0.03(S)	0.015(S)
TGC	1(S)	0.5(S)	1(S)	2(S)	4(I)	2(S)
SXT	≤20(S)	≤20(S)	≤20(S)	≤20(S)	≤20(S)	≤20(S)

Abbreviations: MIC, minimal inhibitory concentration; MEM, meropenem; IPM, imipenem; ETP, ertapenem; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; CXM, cefuroxime; FEP, cefepime; CTX, cefotaxime; CZA, ceftazidime-avibactam; CSL, cefoperazone/sulbactam; TZP, piperacillin-tazobactam; AMC, amoxicillin/clavulanic acid; AMK, amikacin; LVX, levofloxacin; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole.

Enhanced Susceptibility to CZA-Induced Resistance in Isolates with bla_{KPC-12} Compared to bla_{KPC-2} and bla_{KPC-3}

As KPC-12 did not exhibit superior efficacy against ceftazidime-avibactam compared to KPC-2, we further explored the phenotypic differences in CZA-induced resistance among KPC-2, KPC-3, and KPC-12. After five passages, the MIC values of CZA for DH5α::pACYC184-bla_{KPC-2} and DH5α::pACYC184-bla_{KPC-3} increased by 4 folds and 8 folds, respectively (Figure 1A). Notably, a significant 16-fold increase in CZA MIC was observed for DH5α::pACYC184-bla_{KPC-12}. Following five subsequent passages (Figure 1B), the MIC value of CZA for DH5α::pACYC184-bla_{KPC-2} increased from 4/4 µg/mL to 8/4 µg/mL. No change was observed in the MIC of CZA for DH5α::pACYC184-bla_{KPC-3}. However, the MIC of CZA for DH5α::pACYC184-bla_{KPC-12} increased 4 folds, from 32/4 µg/mL to 128/4 µg/mL (Figure 1). The real-time quantitative PCR (RT-qPCR) results indicated that the expression of bla_{KPC-12} in the tenth generation was up-regulated by more than 2-fold (Figure 1C). Taken together, these results suggest that with the widespread use of CZA for treating CRE infections in clinical settings, isolates carrying bla_{KPC-12} are more likely to develop resistance to CZA than those carrying bla_{KPC-2} and bla_{KPC-3}.

To elucidate the molecular mechanism, we used relevant inducible mutants as templates to amplify the DNA fragments encoding bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-12}, and Sanger sequencing was performed to identify the mutation sites. As shown in Figure 5A, a 1-bp deletion was observed at position 859 in bla_{KPC-12} after exposure to CZA for 10 generations. Therefore, this deletion prevented the termination of translation in bla_{KPC-12} might explain why isolates carrying bla_{KPC-12} were more susceptible to CZA resistance (Figure 5B). Interestingly, there was a 6-bp insertion (nucleotide position 492–497) in bla_{KPC-2} and bla_{KPC-3} after the relevant isolates were exposed to CZA for 10 generations (Figure 5A), resulting in 2-amino acid insertions at Ambler positions 169E and 170 L (Figure 5B). However, it is important to note that this nucleotide insertion is not present in bla_{KPC-12}. Overall, the above results suggest that KPC-12 could evolve a unique resistance mechanism for CZA, which is quite different from those of KPC-2 and KPC-3.

Discussion

Ceftazidime-avibactam, which combines ceftazidime with a novel synthetic *β*-lactamase inhibitor avibactam, has become one of the few effective antimicrobial regimens available to combat severe (*Klebsiella pneumoniae* carbapenemase producing *Klebsiella pneumoniae*, KPC-KP) infections. However, resistance to CZA in KPC-KP has been increasingly reported, with even outbreaks being reported in intensive care units[29] and with a significant impact on mortality.[30] There were three principal mechanisms of resistance to CZA have been proposed: (i) overexpression of bla_{KPC} gene accompanied by loss of outer membrane porin, (ii) co-production of metallo-*β*-lactamases, or (iii) specific mutations of bla_{KPC-2} or bla_{KPC-3} within the Ω loop (Amber positions 164_{Arg} to $179_{Asp)}$, which is an essential and conserved active site of class A *β*-lactamases.[31,32] Mutations in the Ω loop region of the KPC enzyme represents the most widespread mechanism, and moreover associated with important diagnostic problems. In fact, these mutated enzymes may go undetected by major carbapenemase detection assays constituting an important problem in active surveillance programs in hospitals.[33,34] In this study, we characterized a CZA-resistant ST11-KL47-type *K. pneumoniae* strain that produces KPC-12 carbapenemase, conferring reduced carbapenem resistance in a sputum specimen. In this study, we found that the L169M variant of KPC-2 exhibits diminished carbapenemase activity. Previous reports have also shown that mutations in the Ω loop can lead to increased CZA resistance and reserve susceptibility to carbapenems.[35–38] KPC-2 variants, such as KPC-33, and KPC-3 variants, such as KPC-39, may enhance ceftazidime affinity and restrict avibactam binding via mutations in the Ω loop followed by D179Y and A172T.[35,36] However, the transformant carrying bla_{KPC-12} exhibited only a 1-fold increase in the MIC of CZA compared to the transformant carrying bla_{KPC-2}. This finding indicates that the *K. pneumoniae* YFKP-97 strain may possess additional mechanisms for resistance to CZA. We mentioned that *K. pneumoniae* YFKP-97 harbored three copies of bla_{SHV-12} in the plasmids pYFKP-97_a and pYFKP-97_b. It has been reported that the expression level of bla_{SHV-12} was much higher in the CZA-resistant strains than that in the CZA-sensitive strains, and the presence of multicopy bla_{SHV-12} was responsible for the reduced sensitivity to CZA.[39] Therefore, we believe that coexistence of multicopy bla_{KPC-12} and bla_{SHV-12} contributes to the development of CZA resistance in the *K. pneumoniae* YFKP-97 strain.

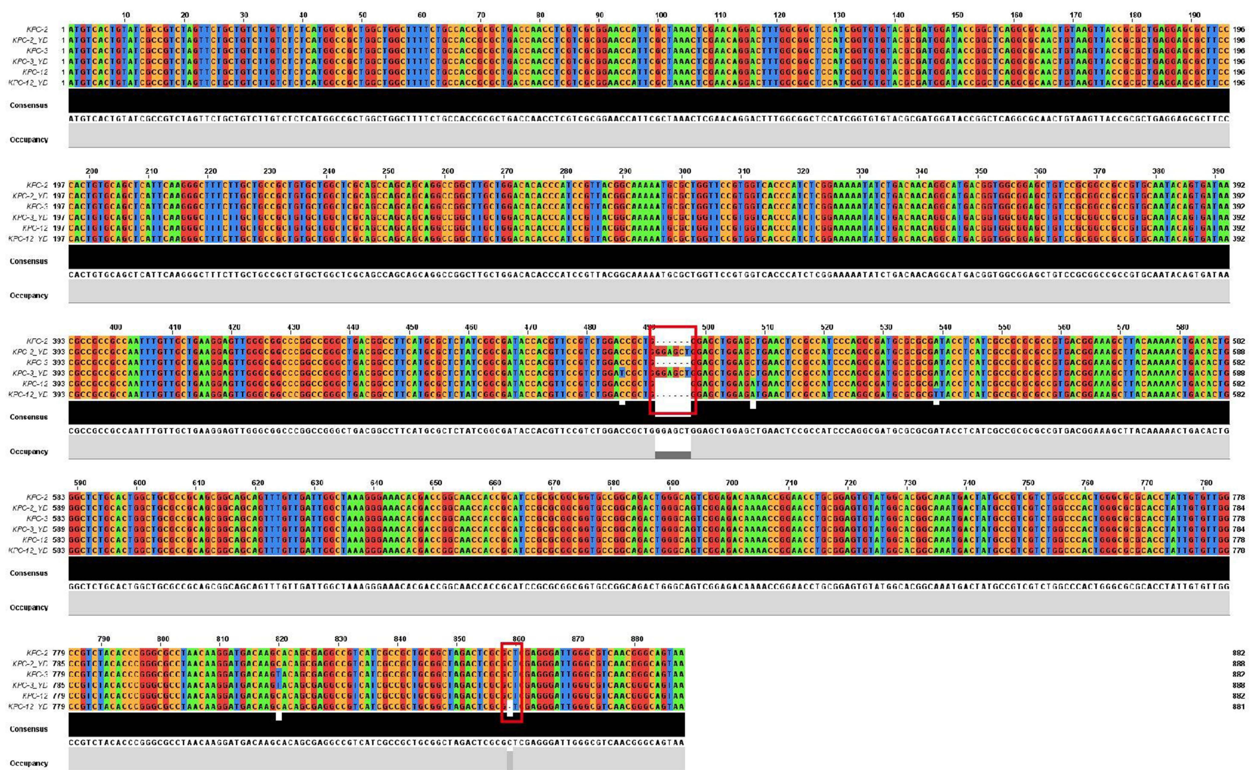


Figure 5 (**A**) The nucleotide sequence alignments of *bla*$_{KPC-2}$, *bla*$_{KPC-3}$ and *bla*$_{KPC-12}$ in relevant strains before and after 10 generations. The differences in the nucleotide sequence were highlighted by using red frames. (**B**) The amino acid sequences alignments of KPC-2, KPC-3 and KPC-12 in relevant strains before and after 10 generations. The differences in the amino acid sequence were highlighted by using red frames. The suffix symbol of "YD" represents the strains obtained after 10 generations of induction with CZA.

Previous studies have shown that the development of resistance to CZA often occurs during prolonged CZA-based treatment.[40–43] Shen et al reported that KPC-112 conferring resistance to CZA could be induced by CZA treatment.[41] Two CZA-resistant carbapenem-susceptible *K. pneumoniae* strains carrying a novel KPC-2 variant, KPC-97, were isolated from a patient treated with CZA in Zhejiang, China.[42] Li et al reported the emergence of a novel KPC-2

variant, KPC-74. This variant was found to confer resistance to CZA in one patient after CZA exposure.[43] It is worth noting that, in our study, KPC-12 was identified in a CRKP strain isolated from a patient infected with *K. pneumoniae* but had never been exposed to CZA treatment. In 2019, Gaibani et al also reported three KPC-KP strains isolated from patients who had not received prior CZA-based treatment, one of which possessed the D179Y mutation in KPC-2.[44] Infections sustained by CZA-resistant patients who was not treated with CZA have been recently reported indicating intra-hospital diffusion of these difficult to treat strains.[29] Considering the high incidence of KPC-KP strains in our country,[6] it is plausible that the patient might acquire the *bla*$_{KPC-12}$ carried pathogen via horizontal transmission during hospitalization. Furthermore, it is noteworthy that *K. pneumoniae* YFKP-97 was isolated from a patient who had been administered empirical anti-tuberculosis treatment. Thus, the destruction of the normal microbial community structure due to the selective pressure exerted by broad-spectrum agents might lead to the emergence of antibiotic-resistant *K. pneumoniae* carrying a novel KPC. Based on our findings, it is essential to be vigilant against the occurrence of CZA-resistant *Enterobacterales* even in patients who have not been previously explored for CZA.

With the widespread use of CZA in clinical practice, although *K. pneumoniae* YFKP-97 was isolated from a patient who had not received prior CZA treatment, we evaluated the risk of induced resistance of KPC-12 cells to CZA in vitro. To the best of our knowledge, this is the first study to compare the inducible CZA resistance patterns in KPC-2, KPC-3, and KPC-12 strains. As mentioned above, the isolate harboring KPC-12 was more prone to evolve into a CZA-resistant phenotype than KPC-2 and KPC-3. Sequence screening revealed that the deletion at position 859, which prevented transcription termination of *bla*$_{KPC-12,}$ could be the reason for the observed phenotypes. Based on our results, we should not only be alert to the occurrence of novel KPC variants exhibiting resistance to CZA but also be vigilant for CZA treatment failure due to the KPC variants being exposed to CZA.

In the United States, ST258 is the predominant KPC-associated CRKP clone.[45] ST11, a variant of ST258, significantly contributes to the dissemination of *bla*$_{KPC}$-positive *K. pneumoniae* in China.[46] It has been reported that there is a sub-clonal shift in the dominant clone ST11 CRKP. Phylogenetic reconstruction revealed that the hypervirulent ST11-KL64 strain was derived from an ST11-KL47-like ancestor through recombination.[47] In the present study, *K. pneumoniae* YFKP-97 belonged to the ST11-KL47 type, which is known to be a prevalent capsular locus in CRKP strains.[48]

The KPC-encoding gene *bla*$_{KPC}$ is regularly present in a single copy of a plasmid or chromosome. Previous reports have shown that KPC-producing isolates contain different levels of *bla*$_{KPC}$ because of multiple *bla*$_{KPC}$ carried plasmids, multiple copies of the *bla*$_{KPC}$ gene located in the same plasmid, or deletions directly upstream of the *bla*$_{KPC}$ gene.[49,50] Herein, we report the discovery of two copies of *bla*$_{KPC-12}$ in an IncFII (pHN7A8) plasmid. Next, we found that *bla*$_{KPC-12}$ copy number can affect the level of KPC production, which contributes to an increase in ceftazidime MIC. Our analysis may partially explain the complex pathways involved in KPC-12 mediated carbapenem resistance phenotype.

In conclusion, this variant of KPC-2, KPC-12, can reverse carbapenem resistance similar to other variants, raising a challenge for routine carbapenemase detection assays. It is extremely important for clinicians to monitor the susceptibility of *bla*$_{KPC}$ harbored strains to ceftazidime-avibactam, even if the patient has not been treated with ceftazidime-avibactam. In addition, it is vital to detect KPC variants at an early stage during therapy to prevent treatment failure, because strains carrying *bla*$_{KPC-12}$ are more prone to evolving CZA-resistant phenotypes than those carrying *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$.

Ethics

The research protocol was approved by the Ethics Committee of Shanghai Pulmonary Hospital (K21-371Y). We have shared the article with the 80-year-old male patient, and he is fully aware of the content of the article it is published. Therefore, we confirm that the 80-year-old male patient gave consent for his details to be published. Guidelines outlined in the Declaration of Helsinki were followed.

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Disclosure

We declare no conflicts of interest.

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