Annals of Clinical Microbiology and Antimicrobials

RESEARCH

Open Access

Ceftazidime-avibactam treatment dilemma of bla_{KPC-2}-containing *Klebsiella pneumoniae* due to the development of co-existence of mixed strains carrying bla_{KPC-2} or bla_{KPC-33} in lung transplant recipients

Zichen Lei[1,2,3†], Ziyao Li[1,2,3,4,5†], Yulin Zhang[2,3], Lingbing Zeng[6], Yongli Wu[2,3,4], Feilong Zhang[2,3,4,7], Xinrui Yang[2,3,4], Xinmeng Liu[2,3,7], Qi Liu[1,2,3], Yiqun Ma[2,3,7] and Binghuai Lu[1,2,3,4,7*]

Abstract

Background Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a significant threat to immunocompromised populations, including lung transplant recipients. This study investigates mixed CRKP strains carrying either bla_{KPC-2} or bla_{KPC-33} following ceftazidime-avibactam (CAZ/AVI) exposure, particularly in the context of lung transplantation. Mixed CRKP strains with shifting resistance phenotypes were frequently identified in patients exposed to CAZ/AVI. We aimed to elucidate the transitional state of bla_{KPC} variants by selecting CAZ/AVI-sensitive and -resistant CRKP strains from a lung transplantation patient.

Methods The bla_{KPC}-variant-carrying CRKP strains were collected from lung transplant recipients exposed to CAZ/AVI in less than two years. Antibiotic susceptibility testing (AST) was conducted using microbroth dilution, and whole-genome sequencing (WGS) was used to identify genotypes and resistance mechanisms. Limiting dilution, drop-plate, and in vitro induction experiments determined bla_{KPC}-variant changes during CAZ/AVI administration. qPCR primers/probes were designed to identify bla_{KPC-2} mutations.

Results Among 104 lung transplant recipients infected by bla_{KPC}-harboring CRKP strains and receiving CAZ/AVI, 10 (9.6%) experienced changing resistance phenotypes. The limiting dilution method found that Patient 10's CRKP strains carried either bla_{KPC-2} or bla_{KPC-33}. The drop-plate experiment showed differing growth patterns on CAZ/AVI mediums. The in vitro induction experiment demonstrated shifting from bla_{KPC-2} to bla_{KPC-33}.

Conclusions The study identified a "transitional state" of the mixed CRKP strains carrying either bla_{KPC-2} or bla_{KPC-33} in CAZ/AVI-exposed patients. Molecular diagnostics are crucial for identifying mixed strains and the transitional state of bla_{KPC} variants, guiding treatment decisions in this complex landscape.

†Zichen Lei and Ziyao Li contributed equally to this work.

*Correspondence:
Binghuai Lu
zs25041@126.com

Full list of author information is available at the end of the article

© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

Keywords Mixed strains carrying different bla_{KPC} variants, bla_{KPC-2}, bla_{KPC-33}, Ceftazidime/Avibactam, Carbapenem-resistant *Klebsiella pneumoniae*, Lung transplant recipients

Introduction

The incidence and impact of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections represent a significant concern in clinical settings, particularly among immunocompromised populations, e.g., lung transplant recipients [1]. CRKP represented the challenge posed by multidrug-resistant organisms, with its ability to evade standard antimicrobial therapies and facilitate outbreaks in healthcare environments. The vulnerability of lung transplant recipients to such infections underscores the urgent need for advanced diagnostic methodologies, effective antimicrobial stewardship, and innovative therapeutic strategies.

K. pneumoniae carbapenemase (KPC)-2 and-3 were predominant among CRKP strains worldwide [2]. Longitudinal data from a comprehensive study conducted between 2012 and 2016 revealed that bla_{KPC-2} is most prevalent among CRKP in China, comprising 76.5% (919/1201) of cases [3]. The novel β-lactam/β-lactamase inhibitor combination, ceftazidime-avibactam (CAZ/AVI), received approval from the FDA in February 2015 and has been available in Chinese market since May 21, 2019 [4]. Avibactam can inhibit a broad spectrum of β-lactamases, including class A (KPC), class C (AmpC), and class D (OXA-48-like), and restore the efficacy of ceftazidime against resistant bacterial strains [5].

Following its market entry, CAZ/AVI has been advocated as the primary therapeutic agent against bla_{KPC}-producing *K. pneumoniae* in China [6]. Nevertheless, an increase in resistance post-administration has been observed, with resistant bla_{KPC} variants emerging [7]. Recent studies have identified various mechanisms of CRKP resistance to CAZ/AVI, including mutations from bla_{KPC-2} to bla_{KPC-33}, alterations in porins, impacts from efflux pumps, and changes in plasmid copy numbers carrying the bla_{KPC} gene [8–10]. The previous studies documented an in vivo evolution event leading to the emergence of bla_{KPC-33}, characterized by a 'Ω-loop' D179Y substitution, which conferred resistance to CAZ/AVI while preserving susceptibility to other carbapenems [9, 11]. In summary, KPC-33 is sensitive to carbapenems but resistant to CAZ/AVI, whereas KPC-2 is resistant to carbapenems but sensitive to CAZ/AVI. Therefore, the ratio of the strains containing different bla_{KPC} genes determines the overall antibiotic resistance phenotype of the mixed bacterial population, which plays a crucial role in the treatment of CRKP. This phenomenon emphasizes the need for continuous surveillance to detect shifts in resistance patterns and understand the ecological and evolutionary dynamics of CRKP within clinical settings [12]. In this study, we focus on the shifting bla_{KPC} variants during CAZ/AVI administration due to the significant clinical challenges posed by bla_{KPC-2}-carrying CRKP in China and the efficacy of CAZ/AVI against these strains.

This study aims to explore the underlying mechanisms of CAZ/AVI resistance development in CRKP strains through the longitudinal analysis of isolates from a lung transplant recipient. In the current study, a mixed strain is defined as two different *K. pneumoniae* strain clones isolated from a single sample, with the co-existence of bla_{KPC-2}-carrying strains and bla_{KPC-33}-carrying strains. Furthermore, we selected a series of CAZ/AVI-sensitive and -resistant CRKP strains consecutively isolated from a lung transplantation patient, because they by coincidence had the following features: (1) The CAZ/AVI resistance of bla_{KPC-2}-carrying CRKP strains was developed after CAZ/AVI exposure, and mediated *via* the emergence of bla_{KPC-33}. (2) The dynamic changing of CAZ/AVI and carbapenem resistance phenotypes in the CRKP strains was identified due to the co-existence of mixed strains of CRKP carrying either bla_{KPC-2} or bla_{KPC-33}, respectively, and changing of the ratio of two genes during the treatment process, which we named as "transitional state" of bla_{KPC} variants. The study will shed light on the potential in-host shifting mechanism between bla_{KPC-2} and bla_{KPC-33}.

Materials and methods

Strain collection and antibiotic sensitivity testing (AST)

The investigation into the prevalence of co-existing bla_{KPC} variants within clinical samples involved the collection of CRKP strains carrying different bla_{KPC}-variants, transiting from bla_{KPC-2} to bla_{KPC-33}, from lung transplantation patients subjected to CAZ/AVI treatment between Sep 2021 and Apr 2023. Demographic variables, including gender, age, and antibiotic administration history, were retrospectively extracted from medical reports.

K. pneumoniae strains were identified by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany). AST was performed using the Vitek-2 system with N335 susceptibility cards for meropenem, ceftazidime, aztreonam, and imipenem. MICs of CAZ/AVI were determined using the microdilution broth method (bio-KONT, Ltd. China), with interpretation based on CLSI-recommended [13]. The presence of bla_{KPC} was verified using the GeneXpert system, and positive cases underwent bla_{KPC} genotyping through polymerase chain

reaction (PCR) and Sanger sequencing with specific primers: F-CGGTGGTGGGCCAATAGATG and R-TCG TCAACCAGACCCGTTTT.

Whole-genome sequencing (WGS) and bioinformatics analysis

Genomic DNA extraction from strains with shifting bla_{KPC} variants was performed using the QIAamp DNA mini kit from Qiagen. Whole-genome sequences were obtained through Illumina NovaSeq PE150 and nanopore sequencing on MinION flow cells. Filtering removed low-quality sequences and adaptors. The online CGE tool (https://cge.cbs.dtu.dk) was employed for multilocus sequence typing (MLST) analysis and identification of acquired antimicrobial resistance genes, while BLASTn against the NCBI database was used for homologous comparisons.

Gene prediction was performed using Prokka 1.13. Antimicrobial resistance genes, MLST, and species identification were analyzed using Kleborate v2.0.4. Furthermore, Snippy 3.2-dev, Gubbins v2.4.1, SNP-sites v2.5.1, RAxMLv8.2.12, and iTOL v5.6 were employed for phylogenetic analysis for high-quality SNPs, filtering recombination events, core polymorphic sites, creating a RAxML tree under the GTR-GAMMA model, and visualization, respectively.

Pulsed-field gel electrophoresis (PFGE) analysis

PFGE was conducted to evaluate the clonal relationships among *K. pneumoniae* strains from Patient 10. Total DNA preparation and PFGE followed established protocols [14]. Strains with a genetic similarity of ≥85% or ≤4 fragment differences in PFGE profiles were classified as the same clone.

Limiting dilution method

The limiting dilution method was used to isolate individual clones of CRKP, each carrying either bla_{KPC-2} or bla_{KPC-33}, as illustrated in Fig. 2.

Briefly, the solution of KP40034-mix strains was prepared to a concentration of 0.5 McFarland, and 110 µl were transferred into the well A1 of a 96-well plate. Simultaneously, except for A1, other 95 wells were added with 100 µl of LB broth. Afterward, 10 µl of KP40034-mix solution was transferred from the preceding well into 100 µl of broth in the subsequent well by using a pipette, from well A1 to well H1, respectively, ensuring thorough mixing. Then, 10 µl solution was transferred from the first column (A1-H1) was transferred into the adjacent column (A2-H2) using an 8-channel pipette. Repeat this process until the last column (A12-H12) was reached, with thorough mixing at each step. After an overnight incubation at 37 °C, OD_{600} absorbance was measured to confirm bacterial growth. Wells showing growth would undergo genomic DNA extraction and Sanger sequencing of the bla_{KPC} gene to validate monoclonality. Monoclonal strains carrying bla_{KPC} were then sub-cultured on LB agar medium for further analysis.

Drop-plate experiment

To validate the co-existence of mixed strains carrying either bla_{KPC-2} or bla_{KPC-33}, we designed a drop-plate experiment, adapted from the agar dilution method but with slight modifications [15]. In brief, five CRKP strains, isolated from Patient 10 using the above limiting dilution method, were selected for further analysis, including one bla_{KPC-2}-harboring strain, two bla_{KPC-33}-harboring ones, and two mixed strains. The McFarland turbidity of each strain was adjusted to 0.5 with 0.45% saline, and serial 10-fold dilutions were prepared for theoretical concentrations ranging from $1\sim1.5\times10^8$ to 10^4 CFU/ml. A 4-µl fully mixed bacterial suspension was spotted onto designated points on six LB agar medium plates supplemented with different CAZ/AVI levels, as depicted in Fig. 3A. After storage at room temperature for 30 min, the plates were incubated at 37℃ overnight to observe bacterial droplet growth.

Following CAZ/AVI breakpoints for a dosage regimen of 2.5 g every 8 h (4:1) administered over 2 h, MICs≤8/4 mg/L and ≥16/4 mg/L were defined as susceptible and resistant, respectively [16]. Additionally, we calculated the AUC (0-τ) values of ceftazidime and avibactam as 289.0 and 42.1 µg·h/mL, respectively, by using the pharmacokinetic formula [17]. The average therapeutic concentrations of ceftazidime and avibactam were 36 and 5 µg/mL, respectively, with a ratio of 7.2 to 1. Therefore, we set the CAZ/AVI levels in LB plates to reflect the actual treatment scenario of samples isolated from the respiratory tract: 0, 8/4, 16/4, 32/4, 48/4, and 72/4 mg/L, respectively.

Growth assay and in vitro induction experiment

To emulate the in vivo phenotypic transition from CAZ/AVI-sensitive to resistant states *via* the mutation of bla_{KPC-2} to bla_{KPC-33} under antimicrobial pressure, an in vitro CAZ/AVI induction resistance experiment was conducted over four days, by using strains KP40034-2 S (solely harboring bla_{KPC-2} and sensitive to CAZ/AVI) and KP40034-mix (containing both bla_{KPC-2}-carrying strains and bla_{KPC-33}-carrying strains), as illustrated in Fig. 4A.

The KP40034-2 S and KP40034-mix strains were incubated overnight at 37 °C in 5 mL LB broth. Subsequently, a 50-µL aliquot from each culture, adjusted to 0.5 McFarland turbidity, was transferred into another tube containing 5 mL fresh LB broth with varying CAZ/AVI concentrations. CAZ/AVI levels and ratios were set at 36/4 mg/L, 48/4 mg/L, 60/4 mg/L, and 72/4 mg/L, respectively, in accordance with a previous study [17].

The experiment was conducted over 4 days. On the first day, the tubes were inoculated with KP40034-mix and KP40034-2 S cultures. For the following three days, 50 µL of the culture from the previous day was utilized to inoculate the freshly prepared LB broth containing CAZ/AVI at different concentrations [18].

After that, a 20-µL aliquot was pipetted for qPCR to ascertain if there was the co-existence of bla_{KPC-2} to bla_{KPC-33}. A 100-µL aliquot, adjusted to 0.5 McFarland turbidity, underwent a 10^7-fold dilution on LB agar medium. Single colonies were selected after overnight culture for Sanger sequencing to detect the 532nd position mutation of the bla_{KPC-2} gene. Additionally, MIC results of CAZ/AVI for the induced strains were determined.

Real-time quantitative PCR (RT-qPCR) probe for bla_{KPC-2} and bla_{KPC-33}

To detect the co-existence of bla_{KPC-2} and bla_{KPC-33} in mixed strains, we developed two qPCR probes targeting the 532nd bp position of the bla_{KPC-2} gene. Relative quantification was performed using the $2^{-\Delta Ct}$ method, as previously described [19]. Sequences of bla_{KPC-2} and bla_{KPC-33} genes from *K. pneumoniae* were obtained from GenBank (CP133867.1 and NG_056170.1). Primer Express software was utilized to design Allele-Refraction Mutation System Polymerase Chain Reaction (ARMS-PCR) primers and consensus probes, which included a mismatch at position −3 from the 3' end to enhance specificity. Locked nucleic acid (LNA) was incorporated, and ARMS-PCR primers had a higher Tm than the forward primers (Supplemental Table 1; LNA denoted by +N). PCR amplification was carried out in two self-contained 25 µL reactions (tube A for bla_{KPC-2} and tube B for bla_{KPC-33}) with dNTPs, buffer, $MgCl_2$, Reverse consensus primer, Taqman consensus probe, and Taq DNA polymerase. Thermal conditions included 1 cycle at 95 °C for 5 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 35 s (fluorescence collection).

The probes effectively detected targeted genes in mixed strains, even after a 10^9-fold dilution from the 0.5 McFarland turbidity level, ensuring separate detection of both bla_{KPC-2} and bla_{KPC-33} genes, even at a ratio as high as 10^5 to 1.

Results

Clinical features of patients receiving the treatment with CAZ/AVI

A total of 104 lung transplantation recipients were enrolled in the present study, who were infected by bla_{KPC}-harboring CRKP and subjected to CAZ/AVI treatment. In them, CRKP strains isolated from 10 patients (9.6%) exhibited continually changing resistance phenotypes against CAZ/AVI and carbapenems due to recurrent shifting of bla_{KPC} variants between bla_{KPC-2} and bla_{KPC-33}.

The antibiotic administration of these 10 patients, their demographic characteristics, and the molecular features of 10 CRKP strains, were shown in Fig. 1A/C. Briefly, they were aged from 48 to 85 years, with a median age of 66 years, with 80% being male (8/10). By comparison, the median age of all 104 lung transplantation patients was 62 years, with 81% being male (84/104). Following CAZ/AVI administration at the recommended dosage for a median of 9 days (range: 3 to 33 days), the daughter strains changed from CAZ/AVI-sensitive to -resistant. Subsequent treatment with meropenem or imipenem led to a reversal of CAZ/AVI sensitivity.

WGS of CRKP strains and analysis

The first non-bla_{KPC-2} strain isolated from each of the 10 patients was selected for WGS, marked with purple circles in Fig. 1A. WGS analysis revealed these strains possessed distinct genetic backgrounds, exhibited no homology, and harbored diverse antibiotic resistance genes, as illustrated in Fig. 1C.

Emergence of the mix strains carrying bla_{KPC-2} and bla_{KPC-33} in Patient 10

Patient 10, a 66-year-old male lung transplant recipient, was selected for further investigation due to distinctive characteristics outlined in the introduction. Six *K. pneumoniae* strains (KP39926, KP40034, KP40118, KP40264, KP40454, KP40726) were consecutively isolated from Patient 10, with five originating from bronchoalveolar lavage fluid (BALF). The timeline of strain isolation and the patient's antibiotic usage history are depicted in Fig. 1B.

These strains underwent changes in resistance genotypes against CAZ/AVI and carbapenems. AST results indicated they were resistant to most antibiotics except for tigecycline. The new combination of antibiotics, e.g., imipenem/relebactam, meropenem/taniborbactam, cefepime/taniborbactam, meropenem/vaborbactam, cefepime/zidebactam, and meropenem/nacubactam, showed sensitivity to the *K. pneumoniae* strains from Patient 10 (Fig. 1C; Table 1). The strains KP40034, KP40264, and KP40454 exhibited resistance to CAZ/AVI and harbored bla_{KPC} genes, as confirmed by the GeneXpert system.

PFGE and the phylogenetic tree revealed identical bands among all six CRKP strains, indicative of genetic similarity. WGS identified them as ST15 with an IncFII plasmid, signifying close genetic relatedness and sharing common ancestry. WGS and AST demonstrated persistent changes in bla_{KPC} variants and resistance phenotypes to CAZ/AVI and carbapenems.

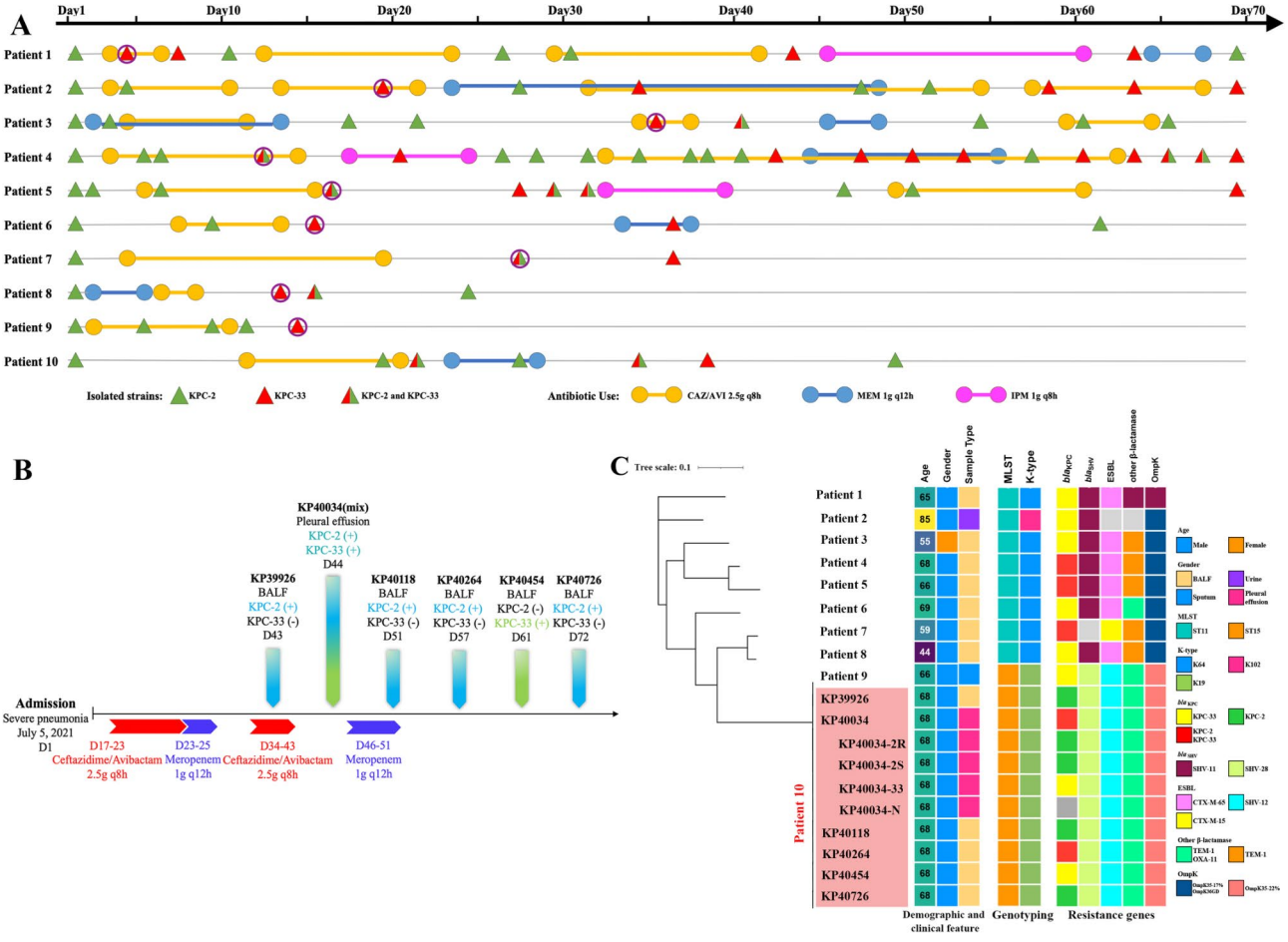

Fig. 1 Strain isolation and antibiotic administration. **(A)** Clinical data for 10 patients in chronological order. ○: First non-bla_{KPC-2}-carrying CRKP strains for further analysis. **(B)** Medical history and antibiotic stewardship of Patient 10 and the corresponding strains. **(C)** Phylogenetic tree and heat map of demographic data of 10 patients, and the molecular characteristics and β-lactamase genes of their corresponding CRKP strains. Notes: CRKP: Carbapenem-resistant *Klebsiella pneumoniae*

Notably, strain KP40034, resistant to both CAZ/AVI and carbapenems, exhibited tiny colonies within the inhibition zone during disc diffusion and E-tests. Further Sanger sequencing of its bla_{KPC} genes revealed there were double peaks at the 532nd position of the bla_{KPC-2} gene (Fig. 1), indicating the co-existence of A and C bases, representing mixed bla_{KPC-2} and bla_{KPC-33} genes. Thus, KP40034 represents mixed CRKP strains producing either bla_{KPC-2} or bla_{KPC-33}.

Limiting dilution method to isolate bla_{KPC}-carrying monoclonal

To isolate monoclonal strains carrying either bla_{KPC-2} or bla_{KPC-33} from the mixed strains KP40034, the limiting dilution method was employed (Fig. 1). Subsequently, the obtained single-clone strains, namely KP40034-2R (bla_{KPC-2}-harboring and resistant to CAZ/AVI), KP40034-2S (bla_{KPC-2}-harboring and sensitive to CAZ/AVI), KP40034-33 (bla_{KPC-33}-harboring and resistant to CAZ/AVI), and KP40034-N (harboring no bla_{KPC} genes and sensitive to CAZ/AVI), underwent sub-culturing, AST experiments, and Sanger sequencing. Despite exhibiting identical morphology, these strains displayed divergent AST results for carbapenem and CAZ/AVI, indicating the presence of either bla_{KPC-2} or bla_{KPC-33}. Besides, these strains can be identified by sequencing. The mixed features of KP40034, representing a "transitional state of bla_{KPC-2} or bla_{KPC-33}," could elucidate its resistance to both carbapenem and CAZ/AVI.

Drop-plate experiment

In a drop-plate experiment, the five CRKP strains were subcultured on LB agar medium supplemented with various CAZ/AVI concentrations, as depicted in Fig. 1B. bla_{KPC-2}-harboring strains grew well on CAZ/AVI-free medium but were rarely seen on CAZ/AVI-supplemented media. Conversely, bla_{KPC-33}-harboring strains and mixed strains grew well on LB agar supplemented with CAZ/AVI, with distinctive colony appearances. The bla_{KPC-33}-harboring strain maintained a round colony,

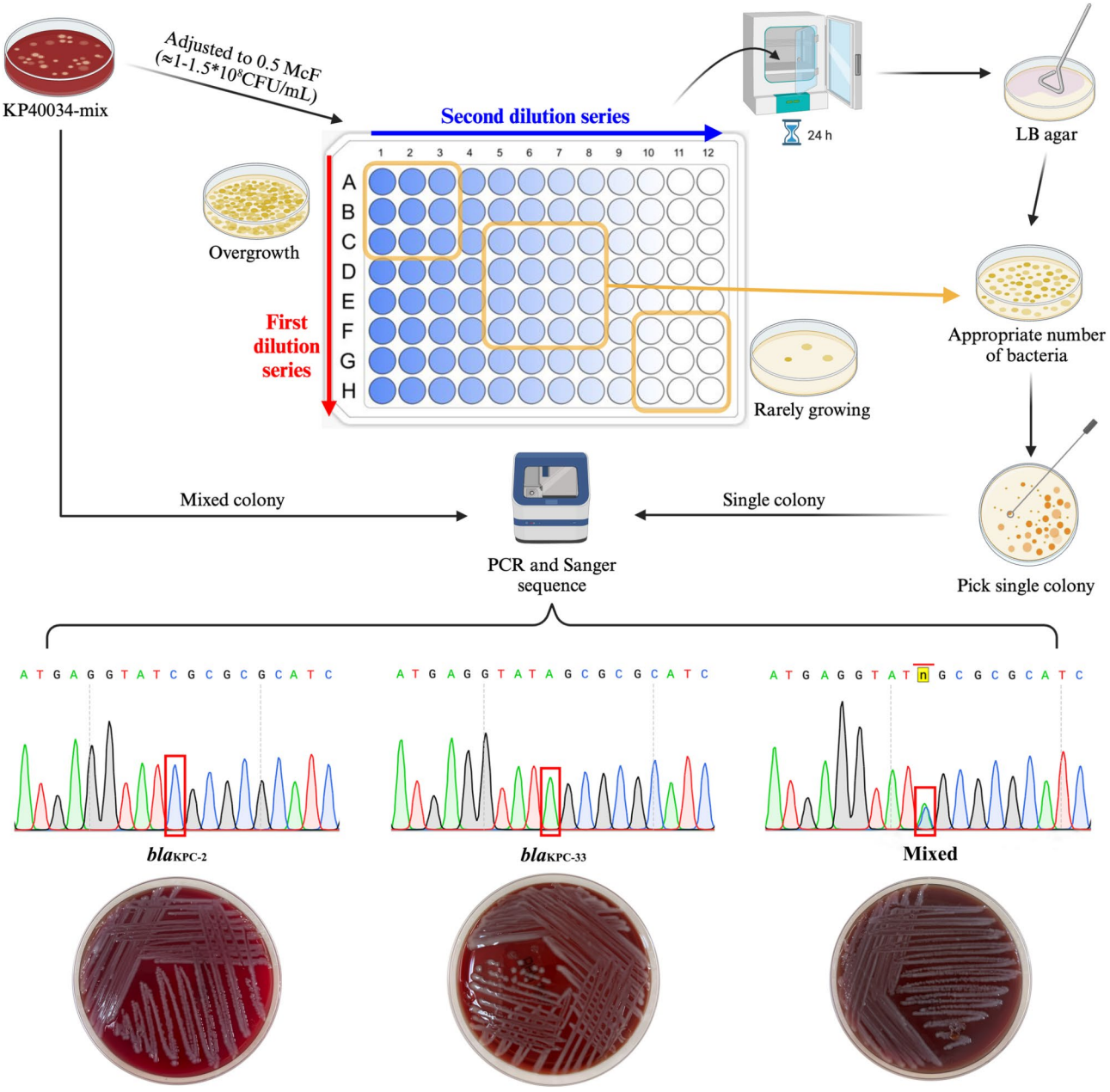

Fig. 2 Limiting dilution and sequencing of mixed strain Isolate bla_{KPC}-carrying monoclonal strains using limiting dilution. Serial 10-fold dilutions in 96-well plates. Select tested strains from the middle zone. Streak them onto LB agar. Select single colonies after overnight incubation at 37℃. Single colonies and mixed KP40034 strains were sequenced to distinguish bla_{KPC} genes. Sanger sequencing of the 523–541 bp region shows 4 colors, Showing the co-existence of A and C at the N site

indicative of a homogeneous clone with a consistent MIC for CAZ/AVI. In contrast, the mixed strains exhibited a spot-like morphology, suggesting partial susceptibility, observed only after dilution to ≥1:100. Additionally, the MIC for CAZ/AVI of mixed strains was lower than that of bla_{KPC-33}-harboring strains. The KPC-33 group showed spots after 10^4 dilutions, while the KPC-mix group showed spots from the original concentration. After the spot morphology could be seen on LB agar, we further conducted serial 10-fold dilution 2–3 times, and no growth was observed.

In vitro induction experiment

In vitro induction experiments were conducted to investigate the impact of CAZ/AVI concentration on the co-existence of bla_{KPC} variants. Starting from the antibiotic treatment dose recommended for lung transplant patients infected with CRKP strains (36/5 mg/L),

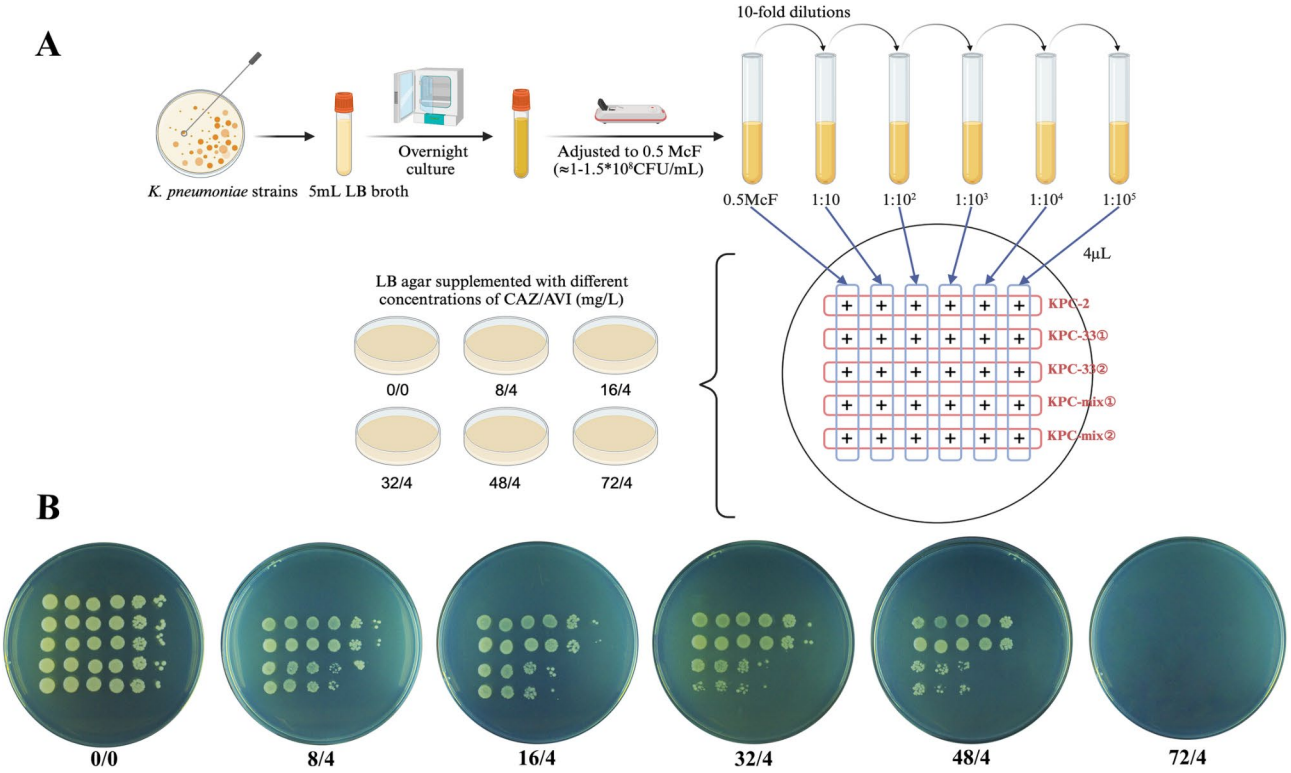

Fig. 3 Drop plate experiments with different dilutions. **(A)** Drop plate experiments: Overnight-grown strains adjusted to 0.5 McFarland turbidity and serially diluted 10-fold. Drops of bacterial suspension were added to 6 LB agar plates with varying CAZ/AVI concentrations at marked positions. **(B)** Results: bla_{KPC-33}-containing strains formed round colonies, while mixed strains showed spot-like morphology

we incrementally elevated the antibiotic concentration until complete bacterial inhibition was achieved. This approach allowed us to explore the dynamic changes in bla_{KPC} variants as the blood CAZ/AVI concentration increased.

As illustrated in Fig. 1B, the results revealed that, at lower CAZ/AVI concentration (≤MIC of mixed strains), the number of bla_{KPC-33}-variant-carrying strains increased over time, signifying a gradual shift from bla_{KPC-2} to bla_{KPC-33} during CAZ/AVI exposure. Misclassification of mixed strains, containing a small number of bla_{KPC-33}-carrying strains, as susceptible could lead to antibiotic treatment failure. Conversely, with increasing CAZ/AVI concentration, bla_{KPC-2}-harboring strains were gradually eliminated. When the CAZ/AVI concentration exceeded the MIC of mixed strains, the antibiotics inhibited both bla_{KPC-2} and bla_{KPC-33}-carrying strains simultaneously, leading to a decrease in OD_{600} absorbance in the bacterial culture solution. However, such a high concentration is difficult to achieve in vivo. The AST results revealed that the MIC of the strains to CAZ/AVI increased as the induction time extended and the CAZ/AVI levels increased.

Discussion

Enterobacteriaceae, and in particular carbapenem-resistant Enterobacteriaceae (CRE), have become increasingly common causes of infections in immunosuppressed populations, e.g., lung transplant recipients [20]. In them, the lung allograft serves as a direct interface with the external environment, contributing to the heightened vulnerability of recipients to microbial invasions. Besides, the emergence and spread of CRKP are concerning, highlighting the urgent need for new antimicrobial agents [21]. The bla_{KPC}-harboring CRKP can be treated with CAZ/AVI, a combination antibacterial agent with the fixed ceftazidime: avibactam ratio of 4:1. In our study, we identified the development of the co-existence of bla_{KPC-2}-carrying strains and bla_{KPC-33}-carrying strains, and their ratio might explain the shift in the antibiotic resistance phenotype of CRKP after exposure to CAZ/AVI. Considering bla_{KPC-2} was the dominant carbapenemase gene in China, many alerts should be raised of the phenomenon of such mixed strains [3].

Our study, including 104 lung transplant patients with bla_{KPC}-harboring CRKP strains, revealed a noteworthy 9.6% experiencing dynamic resistance phenotypes against CAZ/AVI and carbapenems. Notably, the rapid transition from CAZ/AVI sensitivity to resistance occurred within a median of 9 days post-administration,

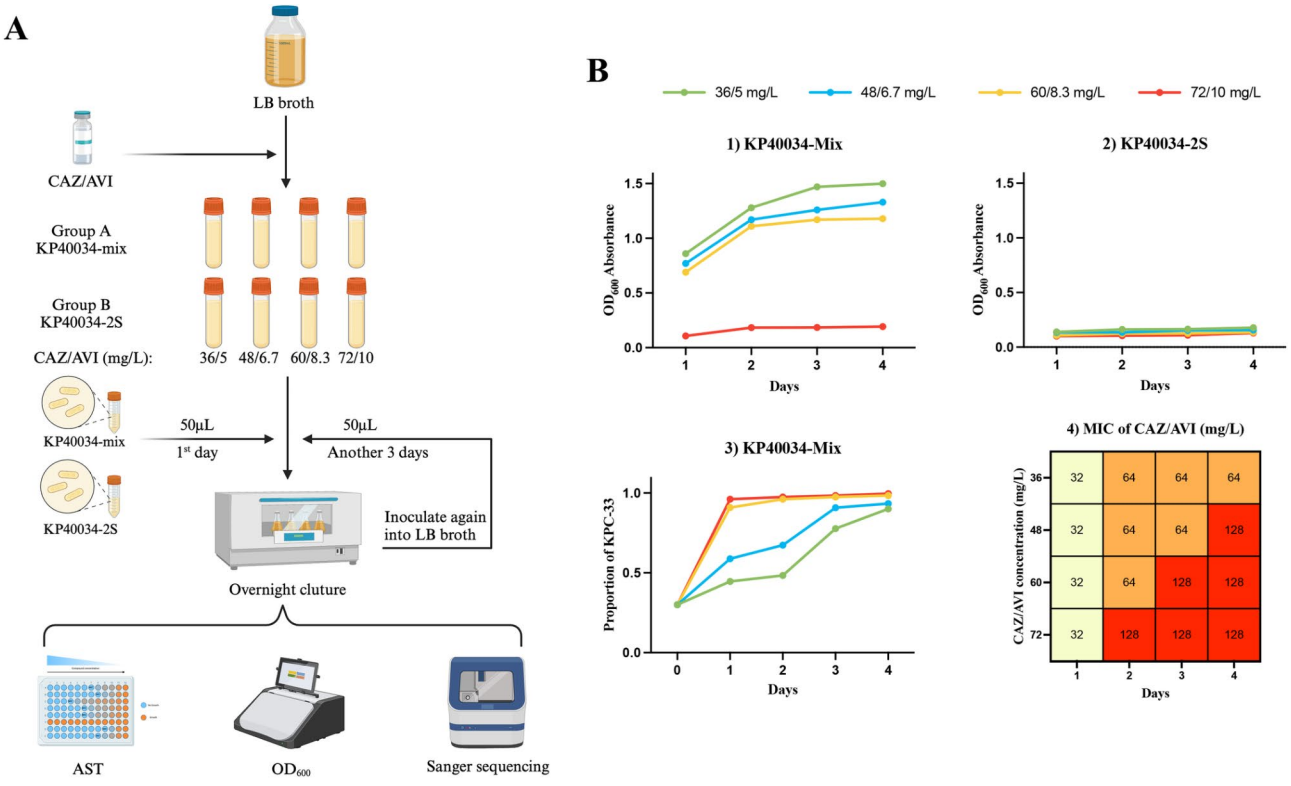

Fig. 4 In vitro induction experiment. **(A)** Procedure: LB broth with different CAZ/AVI concentrations (36/5 mg/L, 48/6.7 mg/L, 60/8.3 mg/L, and 72/10 mg/L, respectively, the same below) divided into two groups. After overnight induction, suspensions were measured for absorbance and sequenced. **(B)** Results: **(1)** OD_{600} of KP40034-mix with CAZ/AVI at differet concentrations: Significant decrease in strains as CAZ/AVI concentration exceeds MIC of mix strains. **(2)** OD_{600} of KP40034-2 S with CAZ/AVI at differet concentrations: Strain count remains low due to susceptibility to CAZ/AVI. **(3)** Proportion of bla_{KPC-33} in KP40034-mix at different CAZ/AVI concentrations: Increases with induction time; insufficient CAZ/AVI doses decrease bla_{KPC-2} but increase bla_{KPC-33}. **(4)** AST of KP40034-mix: Susceptibility to CAZ/AVI increases over time. Notes: AST: antimicrobial susceptibility testing

a timeframe notably shorter than previously documented durations, namely, 10–19 days [9, 22, 23]. These findings carry profound implications for the clinical management of CRKP infections in lung transplant recipients.

The always-changing CAZ/AVI phenotype due to bla_{KPC} mutation might lead to treatment failure and attract more concerns [7]. Studies showed that the aspartic acid at position 179 of the Ω loop in the resistant bla_{KPC-2}-carrying CRKP strain was often replaced with tyrosine, which caused a mutation of bla_{KPC-2} to bla_{KPC-33} [24]. The Ω loop, located at residues 164–179 in class A β-lactamases, is stabilized by salt bridges at Arg-164 and Asp-179. A substitution at residue 179 makes the enzyme bind ceftazidime more easily, increasing its hydrolysis rate. This change reduces avibactam's ability to inhibit the enzyme but restores susceptibility to carbapenems [25–28].

In the study, Patient 10 was treated alternately with CAZ/AVI and meropenem, or both, and 6 strains carrying the bla_{KPC-2}, bla_{KPC-33}, or both, were isolated, and they shared significant homology. Furthermore, in vitro experiment, we noticed the colonies of the strains carrying different bla_{KPC} variants presented different appearances on the MH plate supplemented with CAZ/AVI at different levels. Sanger sequencing saw double peaks at the positive of 532nd bp of bla_{KPC}, showing the KP40034 strain consists of multiple clones, rather than a single colony, with identical morphology but different bla_{KPC} variants. This explained why it showed resistance to both carbapenems and CAZ/AVI. The mixed strains could be further separated into multiple strains by the limiting dilution method. In previous studies, it was reported that bla_{KPC-2} changed to bla_{KPC-33}, and after using meropenem, the bla_{KPC-2} reemerged too [24, 29]. Herein, we first documented the existence of mixed strains carrying different bla_{KPC} variants, as corned here as "transitional state", a state between CAZ/AVI resistance and sensitivity, or "mixed strains".

In this study, two methods were newly designed or modified. To confirm the co-existence of mix strains, each strain carrying bla_{KPC-2} or bla_{KPC-33}, sensitive and resistant to CAZ/AVI, respectively, we designed a drop-plate experiment, adapted from the agar dilution method. The drop plate experiment is based on the concept of diluting a bacterial solution to a predetermined concentration and then dropping it onto an LB agar medium

Table 1 Antibiotic resistance characteristics (MICs, mg/L) and WGS analysis of clinical *K. pneumoniae* strains

Name		MLST	K type	bla_{KPC} gene	CZA	MEM	CAZ	COL	IPM	TGC	ATM	IPM /*R*	MEM /T	FEP /T	MEM /V	FEP /Z	MEM /*N*
Patient 1		ST11	K64	KPC-33	**16**	1	**> 64**	0.25	0.5	2	**> 128**						
Patient 2		ST11	K102	KPC-33	**32**	0.25	**32**	0.25	0.5	0.25	**128**						
Patient 3		ST11	K64	KPC-33	**32**	1	**> 64**	0.25	0.25	**16**	**> 128**						
Patient 4		ST11	K64	KPC-2, KPC-33	**64**	**4**	**> 64**	0.25	0.5	**4**	**> 128**						
Patient 5		ST11	K64	KPC-2, KPC-33	**16**	**8**	**16**	0.5	**32**	0.5	**128**						
Patient 6		ST11	K64	KPC-33	**32**	1	**> 64**	0.25	0.5	**4**	**> 128**						
Patient 7		ST11	K64	KPC-2, KPC-33	**32**	**4**	**> 64**	0.5	1	1	**128**						
Patient 8		ST11	K64	KPC-33	**32**	1	**> 64**	2	0.5	0.5	**> 128**						
Patient 9		ST15	K19	KPC-33	**32**	1	**> 64**	0.25	0.5	**4**	**> 128**						
Patient 10	*KP39926*	ST15	K19	KPC-2	0.5	**16**	**64**	**32**	**8**	2	**> 128**	0.25/4	1/4	1/4	0.5/8	4/4	2/2
	KP40034(mix)	ST15	K19	KPC-2, KPC-33	**64**	**16**	**> 64**	0.5	**16**	1	**> 128**	0.5/4	2/4	8/4	2/8	4/4	2/2
	KP40034-2R	ST15	K19	KPC-2	**32**	**16**	**64**	0.5	**16**	1	**128**	2/4	1/4	2/4	2/8	2/2	2/2
	KP40034-2 S	ST15	K19	KPC-2	0.5	**16**	**64**	0.5	**8**	0.5	**128**	0.5/4	1/4	4/4	1/8	1/1	2/2
	KP40034-33	ST15	K19	KPC-33	**32**	0.5	**64**	0.5	0.25	0.5	**128**	0.125/4	2/4	8/4	1/8	2/2	1/1
	KP40034-N	ST15	K19	-	1	0.5	**64**	0.5	0.25	0.5	**128**	0.25/4	0.5/4	0.5/4	0.125/8	0.25/0.25	0.5/0.5
	KP40118	ST15	K19	KPC-2	1	**16**	**> 64**	1	**8**	1	**> 128**	1/4	1/4	1/4	8/8	2/2	2/2
	KP40264	ST15	K19	KPC-2, KPC-33	**32**	**16**	**> 64**	0.5	**16**	1	**> 128**	0.25/4	1/4	4/4	1/8	2/2	1/1
	KP40454	ST15	K19	KPC-33	**32**	0.25	**> 64**	0.5	0.25	1	**128**	0.5/4	1/4	4/4	1/8	4/4	2/2
	KP40726	ST15	K19	KPC-2	2	**16**	**> 64**	0.5	**16**	1	**> 128**	0.5/4	1/4	4/4	2/8	2/2	2/2

Notes: MIC of tigecycline and colistin was detected using microdilution broth method. Abbreviations: COL, Colistin; CAZ/AVI, Ceftazidime/Avibactam; CAZ, Ceftazidime; ATM, Aztreonam; MEM, Meropenem; IPM, Imipenem; TGC, Tigecycline; IPM/R, Imipenem/relebactam; MEM/T, Meropenem/taniborbactam; FEP/T, Cefepime/taniborbactam; MEM/V, Meropenem/vaborbactam; FEP/Z, Cefepime/zidebactam; MEM/N, Meropenem/nacubactam; MLST, Multi-Locus Sequence Typing, MIC, Minimum inhibitory concentration, WGS, Whole genome sequencing

containing CAZ/AVI. If the bacteria in the solution are single clones, they will be incapable of multiplying when the antibiotic concentration exceeds the MIC of specific CRKP strains to apply CAZ/AVI. As shown in Fig. 1B, the bla_{KPC-33}-harboring strain grew into a round colony. However, the mixed strain showed spot-like morphology due to the inhibition of bla_{KPC-2} strain by CAZ/AVI in LB agar. Even though mixed strain shows two slightly different colony morphology, whether those strains are bla_{KPC-2}- or bla_{KPC-33}-carrying strains can only be identified using sequencing.

Besides, our study underscores the need for enhanced molecular diagnostics, such as the qPCR probes designed to detect specific mutations, to overcome the limitations of AST in identifying the mixed strains. To address this issue, we designed two qPCR probes to detect the mutations of bla_{KPC-2} gene at the position of 532nd bp, but unable to identify other bla_{KPC} variants sharing the mutations at the above position with bla_{KPC-33}, though this rarely occurs clinically.

In the in vitro induction experiment, lower levels of CAZ/AVI induced a transformation of bla_{KPC-2} strains into mixed strains carrying bla_{KPC-33}, showing CAZ/AVI resistance and resulting in clinical treatment failure. Conversely, higher concentrations of CAZ/AVI could eliminate mixed strains that harbor both bla_{KPC-2}-carrying strains and bla_{KPC-33}-carrying strains. However, considering the permeability of ceftazidime from plasma to alveolar epithelial lining fluid cells reported in a previous study as 20.6±8.9% [30], achieving such elevated concentrations in humans poses a challenge, thereby leading to clinical treatment dilemmas. Innovative antibiotic combinations, such as meropenem/vaborbactam or imipenem/relebactam, may offer promising solutions to address this issue.

In conclusion, our study sheds light on the shifting resistance patterns of CRKP receiving CAZ/AVI treatment. The bla_{KPC-33}-harboring CRKP was sensitive to carbapenems but often co-existed with the bla_{KPC-2}-containing strains, thus often leading to meropenem/imipenem treatment failure. The rapid transition between resistance phenotypes, coupled with the emergence of mixed strains carrying different bla_{KPC} variants, necessitates a paradigm shift in our approach to diagnosing and treating CRKP infections. This transitional state underscores the complexity of CRKP infections in those receiving CAZ/AVI. Besides, our study underscores the need for enhanced molecular diagnostics, such as the qPCR probes designed to detect specific mutations, to overcome the limitations of AST in identifying mixed strains.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12941-024-00743-x.

Supplementary Material 1

Author contributions

Project design: LBH, LZC and LZY. Methodology: LZC, ZLB and YXR. Project administration: LBH and ZYL. Data analyses: LZC, LXM, ZFL and LQ. Manuscript writing: LZC, WYL and MYQ. All authors provided final approval of the version submitted for publication.

Funding

This work was supported by Beijing Natural Science Foundation (7242124), the CAMS Innovation Fund for Medical Sciences (CIFMS; No.2021-I2M-1-030)), and the National Natural Science Foundation of China [grant number 82300012].

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

In accordance with the Declaration of Helsinki, the use of the *K. pneumoniae* isolates for research purposes was approved by the ethics committee of the China-Japan Friendship Hospital (2022-KY-054).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Competing interests

The authors declare no competing interests.

Author details

[1]China-Japan Friendship Institute of Clinical Medical Sciences, China-Japan Friendship Hospital, Beijing, China
[2]Laboratory of Clinical Microbiology and Infectious Diseases, Department of Pulmonary and Critical Care Medicine, Center of Respiratory Medicine, National Clinical Research Center for Respiratory Diseases, National Center for Respiratory Medicine, China-Japan Friendship Hospital, Beijing, China
[3]Institute of Respiratory Medicine, Chinese Academy of Medical Sciences, Beijing, China
[4]Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China
[5]Changping Laboratory, Beijing, China
[6]Department of Clinical Laboratory, The First Affiliated Hospital of Jiangxi Medical College, Nanchang University, Nanchang, China
[7]Peking University China-Japan Friendship School of Clinical Medicine, Beijing, China

Received: 31 March 2024 / Accepted: 5 September 2024
Published online: 08 November 2024

References

1. Perez F, El Chakhtoura NG, Papp-Wallace KM, Wilson BM, Bonomo RA. Treatment options for infections caused by carbapenem-resistant Enterobacteriaceae: can we apply precision medicine to antimicrobial chemotherapy? Expert Opin Pharmacother. 2016;17:761–81.
2. Ding L, Shen S, Chen J, Tian Z, Shi Q, Han R, et al. Klebsiella pneumoniae carbapenemase variants: the new threat to global public health. Clin Microbiol Rev. 2023;36:e00008–23.
3. Wang Q, Wang X, Wang J, Ouyang P, Jin C, Wang R, et al. Phenotypic and genotypic characterization of carbapenem-resistant Enterobacteriaceae: data from a longitudinal large-scale CRE study in China (2012–2016). Clin Infect Dis. 2018;67:S196–205.
4. Forest Pharmaceuticals I. Avycaz: ceftazidime-avibactam prescribing information. Forest Pharmaceuticals, Inc Cincinnati, Ohio; 2016.
5. Sharma R, Park TE, Moy S. Ceftazidime-avibactam: a novel cephalosporin/β-lactamase inhibitor combination for the treatment of resistant Gram-negative organisms. Clin Ther. 2016;38:431–44.

6. Zeng M, Xia J, Zong Z, Shi Y, Ni Y, Hu F et al. Guidelines for the diagnosis, treatment, prevention and control of infections caused by carbapenem-resistant gram-negative bacilli. J Microbiol Immunol Infect. 2023.
7. Nichols WW, Bradford PA, Stone GG. The primary pharmacology of ceftazidime/avibactam: microbiology from clinical studies, and development of resistance during treatment. J Antimicrob Chemother. 2023;78:871–92.
8. Hobson CA, Pierrat G, Tenaillon O, Bonacorsi S, Bercot B, Jaouen E, et al. Klebsiella pneumoniae carbapenemase variants resistant to ceftazidime-avibactam: an evolutionary overview. Antimicrob Agents Chemother. 2022;66:e00447–22.
9. Shi Q, Yin D, Han R, Guo Y, Zheng Y, Wu S, et al. Emergence and recovery of ceftazidime-avibactam resistance in Bla KPC-33-harboring Klebsiella pneumoniae sequence type 11 isolates in China. Clin Infect Dis. 2020;71:S436–9.
10. Xu T, Guo Y, Ji Y, Wang B, Zhou K. Epidemiology and mechanisms of ceftazidime–avibactam resistance in Gram-negative bacteria. Engineering. 2022;11:138–45.
11. Wang C, Zhao J, Liu Z, Sun A, Sun L, Li B, et al. In vivo selection of imipenem resistance among ceftazidime-avibactam-resistant, imipenem-susceptible Klebsiella pneumoniae isolate with KPC-33 carbapenemase. Front Microbiol. 2021;12:727946.
12. Zhang R, Liu L, Zhou H, Chan EW, Li J, Fang Y, et al. Nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains in China. EBioMedicine. 2017;19:98–106.
13. Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, Clancy CJ. Infectious Diseases Society of America 2023 guidance on the treatment of antimicrobial resistant gram-negative infections. Clin Infect Dis. 2023:ciad428.
14. Han H, Zhou H, Li H, Gao Y, Lu Z, Hu K, et al. Optimization of pulse-field gel electrophoresis for subtyping of Klebsiella pneumoniae. Int J Environ Res Public Health. 2013;10:2720–31.
15. Elias R, Melo-Cristino J, Lito L, Pinto M, Gonçalves L, Campino S, et al. Klebsiella pneumoniae and Colistin susceptibility testing: performance evaluation for Broth Microdilution, Agar Dilution and Minimum Inhibitory Concentration Test strips and Impact of the Skipped Well Phenomenon. Diagnostics. 2021;11:2352.
16. Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, Clancy CJ. Infectious Diseases Society of America 2023 Guidance on the treatment of Antimicrobial resistant gram-negative infections. Clinical Infectious Diseases; 2023.
17. Shirley M. Ceftazidime-avibactam: a review in the treatment of serious gram-negative bacterial infections. Drugs. 2018;78:675–92.
18. Jiang M, Sun B, Huang Y, Liu C, Wang Y, Ren Y et al. Diversity of ceftazidime-avibactam resistance mechanism in KPC2-producing klebsiella pneumoniae under antibiotic selection pressure. Infect Drug Resist. 2022:4627–36.
19. Ruzin A, Visalli MA, Keeney D, Bradford PA. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in Klebsiella pneumoniae. Antimicrob Agents Chemother. 2005;49:1017–22.
20. Singh N, Wagener MM, Obman A, Cacciarelli TV, de Vera ME, Gayowski T. Bacteremias in liver transplant recipients: shift toward gram-negative bacteria as predominant pathogens. Liver Transpl. 2004;10:844–9.
21. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, et al. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect. 2012;18:413–31.
22. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of ceftazidime-avibactam resistance due to plasmid-borne bla KPC-3 mutations during treatment of carbapenem-resistant Klebsiella pneumoniae infections. Antimicrob Agents Chemother. 2017;61:02097–16. https://doi.org/10.1128/aac.
23. Hemarajata P, Humphries RM. Ceftazidime/avibactam resistance associated with L169P mutation in the omega loop of KPC-2. J Antimicrob Chemother. 2019;74:1241–3.
24. Li D, Li K, Dong H, Ren D, Gong D, Jiang F et al. Ceftazidime-Avibactam resistance in Klebsiella pneumoniae sequence type 11 due to a mutation in plasmid-borne bla KPC-2 to bla KPC-33, in Henan, China. Infect Drug Resist. 2021:1725–31.
25. Levitt PS, Papp-Wallace KM, Taracila MA, Hujer AM, Winkler ML, Smith KM, et al. Exploring the role of a conserved class A residue in the Ω-loop of KPC-2 β-lactamase: a mechanism for ceftazidime hydrolysis. J Biol Chem. 2012;287:31783–93.
26. Shields RK, Nguyen MH, Press EG, Chen L, Kreiswirth BN, Clancy CJ. In vitro selection of meropenem resistance among ceftazidime-avibactam-resistant, meropenem-susceptible Klebsiella pneumoniae isolates with variant KPC-3 carbapenemases. Antimicrob Agents Chemother. 2017;61. https://doi.org/10.1128/aac. 00079 – 17.
27. Gaibani P, Campoli C, Lewis RE, Volpe SL, Scaltriti E, Giannella M, et al. In vivo evolution of resistant subpopulations of KPC-producing Klebsiella pneumoniae during ceftazidime/avibactam treatment. J Antimicrob Chemother. 2018;73:1525–9.
28. Compain F, Arthur M. Impaired inhibition by avibactam and resistance to the ceftazidime-avibactam combination due to the D179Y substitution in the KPC-2 β-lactamase. Antimicrob Agents Chemother. 2017;61. https://doi.org/10.1128/aac. 00451 – 17.
29. Ding L, Shen S, Han R, Yin D, Guo Y, Hu F. Ceftazidime–Avibactam in Combination with Imipenem as Salvage Therapy for ST11 KPC-33-Producing Klebsiella pneumoniae. Antibiotics. 2022;11:604.
30. Boselli E, Breilh D, Rimmelé T, Poupelin J-C, Saux M-C, Chassard D, et al. Plasma and lung concentrations of ceftazidime administered in continuous infusion to critically ill patients with severe nosocomial pneumonia. Intensive Care Med. 2004;30:989–91.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.