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SUPPLEMENT ARTICLE

# Emergence and Recovery of Ceftazidime-avibactam Resistance in *bla*$_{KPC-33}$-Harboring *Klebsiella pneumoniae* Sequence Type 11 Isolates in China

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This is the first report of ceftazidime–avibactam resistance caused by the *bla*$_{KPC-33}$ mutation through the D179Y variant during the treatment of *bla*$_{KPC-2}$-positive *Klebsiella pneumoniae*-related infections in China. The *bla*$_{KPC-33}$-containing *K. pneumoniae* was susceptible to meropenem–vaborbactam, cefepime–zidebactam, tigecycline, and polymyxin B. The *bla*$_{KPC-33}$ gene was located on a 77 551-bp transformable plasmid harboring *qnrS1* and *bla*$_{LAP-2}$. Detecting *bla*$_{KPC-33}$-positive *K. pneumoniae* clinical strains is important for infection control.

**Keywords.** Carbapenem-resistant *K. pneumoniae*; Ceftazidime-avibactam; Meropenem-vaborbactam; *bla*$_{KPC-2}$; *bla*$_{KPC-33}$.

Carbapenemase-producing *Enterobacterales* (CPE)-related infections have been a major public healthcare problem, causing global concerns. Ceftazidime–avibactam has been approved as an effective alternative antibiotic for clinical treatment against *bla*$_{KPC}$- and *bla*$_{OXA-48}$-producing isolates in recent years. However, resistance has gradually emerged along with its wide usage. Herein, we present the first case of the emergence of ceftazidime–avibactam-resistant KPC-33 carbapenemase-producing *K. pneumoniae* in China, and described its molecular and genetic characteristics in detail.

## METHODS

Three multidrug-resistant *Klebsiella pneumoniae* strains, H1, H2, and H3, were isolated from sputum specimens of a hospitalized patient at Huashan Hospital in Shanghai, China. They were identified using MALDI-TOF MS (bioMérieux, Marcy-l'Étoile, France). *Escherichia coli* ATCC 25922 and *bla*$_{KPC-2}$-containing *K. pneumoniae* ATCC 1705 were used as controls for screening carbapenemase genes and testing antimicrobial susceptibility. *Escherichia coli* EC600 and *Salmonella braenderup* H9812 were used as recipients for conjugation experiments and reference markers for pulsed-field gel electrophoresis (PFGE), respectively. The minimal inhibition concentration (MIC) was determined using the reference Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [1]. Quality control and interpretation of the results were also performed according to 2020 CLSI breakpoints [1] for all agents except tigecycline and polymyxin B, which were interpreted using the interpretative criteria according to the Food and Drug Administration and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), respectively. The efflux pump inhibitor phenyl-arginine-β-naphthylamide (25 mg/L) was added into the broth to observe ceftazidime-avibactam MIC variation; no less than a quadruple MIC decrease was considered to be significant.

Conjugation experiments were performed to explore the transferability of the ceftazidime–avibactam resistance using rifampicin-resistant *E. coli* EC600 as a recipient strain. The transconjugants were selected on Luria-Bertani agar plates containing ceftazidime–avibactam (8 mg/L) and rifampicin (200 mg/L), and were verified by PCR for the presence of *bla*$_{KPC}$. Bacterial DNA was digested with *Xba I* and S1-nuclease subjected to PFGE and S1-PFGE, respectively. PFGE was performed using a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA). PFGE patterns were interpreted in accordance with the criteria of Tenover et al. [2] using BioNumerics software version 6.5. Genomic DNAs of *K. pneumoniae* strains were subjected to whole-genome sequencing using Illumina (Illumina, San Diego, CA, USA) short-read sequencing (150-bp paired-end reads). Sequences were trimmed with sickle (GitHub) and de novo assembled using SPAdes 3.12.0. To evaluate and compare the assembly results, Pilon 1.18 was used for base correction. Multilocus sequence typing (MLST) and antimicrobial resistance genes analysis were performed using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/) and ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder-3.2/), respectively.

Clinical characteristics including age, gender, disease recognition, specimen origin and separation time, antibiotic

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exposure and duration time, in-hospital time, and disease prognosiswere systematically extracted from the electronic medical records.

Ethics committee approval was obtained from the institutional review board of Huashan Hospital for these isolates, and verbal informed consent from patient's parents was also accepted and approved.

## RESULTS

The first carbapenem-resistant *K. pneumoniae* (H1, $bla_{KPC-2}$ positive) was isolated from the sputum of a 42-year-old man, who underwent an endoscopic-assisted transsphenoidal approach resection for prolactin-producing pituitary macroadenoma 3 days after hospitalization. Subsequently, ceftazidime–avibactam and tigecycline were used to replace the empirical meropenem regimen for anti-infection. However, after using ceftazidime–avibactam for 16 days, the patient's body temperature rose again, concurrent with the symptoms of severe pulmonary infection. The second carbapenem-resistant *K. pneumoniae* strain (H2, $bla_{KPC-33}$ positive) was isolated on day 45 from the sputum. Considering the side effects and patient's economic condition, imipenem, amoxicillin–clavulanic, and gradually reduced doses of polymyxin B were used instead. Unfortunately, recurrent pneumonia emerged on day 63, and the third carbapenem-resistant *K. pneumoniae* (H3, $bla_{KPC-2}$ positive) was isolated from the sputum. Clinical and microbiologic details, timelines, and antibiotic therapies used are summarized in Figure 1.

H1, H2, and H3 were $bla_{KPC}$-positive isolates, and were highly resistant to β-lactams, aminoglycosides, quinolones, and sulfanilamides (Table 1). H1 and H3 were highly resistant to carbapenems and susceptible to ceftazidime–avibactam; while H2 ($bla_{KPC-33}$ positive) was resistant to ceftazidime–avibactam but susceptible to imipenem with MIC = 0.25 mg/L. Further, cefepime–zidebactam and meropenem–vaborbactam, as well as tigecycline and polymyxin B, showed good in vitro activities against all 3 isolates. The addition of phenyl-arginine-β-naphthylamide did not change the MIC of ceftazidime–avibactam. The plasmid harboring $bla_{KPC-33}$ from strain H2 was successfully transferred into the EC600 *E. coli* recipient strain, making the transconjugants resistant to ceftazidime–avibactam, ertapenem, quinolone, and some cephalosporins (Table 1).

PFGE showed that the H1, H2, and H3 strains were highly homologous, showing a consistent fingerprint spectrum. Per the whole-genome sequencing analysis, H2 belonged to the ST11 type, harboring the *aadA2b*, *rmtB*, *qnrS1*, $bla_{LAP-2}$, $bla_{SHV-12}$, $bla_{KPC-33}$, $bla_{CTX-M-65}$, $bla_{TEM-1B}$, *dfrA14*, *fosA*, *sul2*, and *tet*(*A*) resistance genes. $bla_{KPC-33}$ gene emergence was due to a single base mutation at G532T, causing the D179Y amino acid mutation of the $bla_{KPC-2}$ gene. The plasmid harboring $bla_{KPC-33}$ was a 77 551-bp conjugative plasmid (plasmid pKPC-H2; average GC content, 53.79%). In this plasmid, *qnrS1*, $bla_{LAP-2}$, and $bla_{KPC-33}$ were located at the same gene island. Mobile elements such as Tn3-family transposons, TnAs1, IS26, ISKpn27, ISKpn6, ISKpn19, and IS3 were also distributed densely around the resistant genes; these can cluster and combine with resistance genes to transfer multiple resistance of plasmids. pKPC-H2 was 100% covered and showed 99.66% identity when mapped to the plasmid (GenBank accession no. CP050279.1) with an 18 962-bp fragment deletion in the downstream region, containing the repeat resistance genes *qnrS1* and $bla_{LAP-2}$. Additionally, a single-nucleotide deletion at position 86 of the OmpK35-encoding gene was found, producing a frameshift mutation and a premature stop codon in the coding sequence.

## DISCUSSION

As one of the most effective antibiotics against $bla_{KPC-2}$-positive strains, ceftazidime–avibactam has been widely used following its approval in China on May 21, 2019. However, with its ever-growing clinical application, the rate of ceftazidime–avibactam resistance in carbapenem-resistant *K. pneumoniae*-infected patients has reached approximately 10% [3]. The basic resistance mechanisms are as follows: (1) KPC or OXA-48

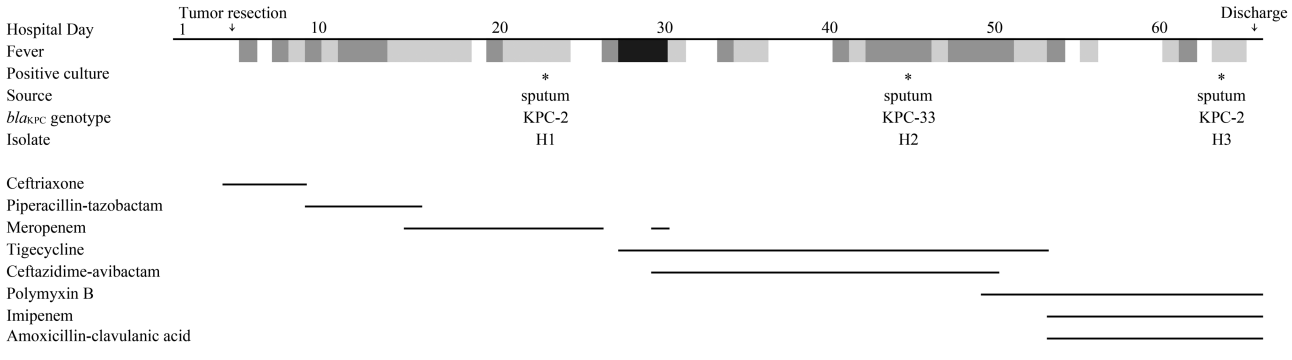


**Figure 1.** Time courses of infection and treatment of the patient with multidrug-resistant *K. pneumoniae* infection. The blocks from dark to light indicate high, mediate, low-grade fever, and normal temperatures.

**Table 1. Minimal Inhibitory Concentrations (MICs) of *K. pneumoniae* Strains H1, H2, H3, and the $bla_{KPC-33}$-Positive *E. coli* Transconjugant of H2**

| | MIC (μg/mL) for: | | | | |
|---|---|---|---|---|---|
| Antimicrobial agents | *K. pneumoniae* H1 ($bla_{KPC-2}$) | *K. pneumoniae* H2 ($bla_{KPC-33}$) | *K. pneumoniae* H3 ($bla_{KPC-2}$) | Transconjugant *E. coli* H2-EC600 ($bla_{KPC-33}$) | *E. coli* EC600 |
| Amikacin | >128 | >128 | >128 | ≤1 | ≤1 |
| Cefazolin | >32 | >32 | >32 | 8 | 4 |
| Cefuroxime | >32 | >32 | >32 | >32 | 16 |
| Ceftriaxone | >32 | >32 | >32 | 8 | ≤0.5 |
| Ceftazidime | >32 | >32 | >32 | >32 | 0.5 |
| Cefepime | >128 | >128 | >128 | 4 | 0.125 |
| Aztreonam | >128 | >128 | >128 | 2 | ≤1 |
| Imipenem | 32 | 0.25 | 64 | 0.125 | 0.125 |
| Meropenem | >64 | 4 | >64 | 0.03 | ≤0.03 |
| Ertapenem | >64 | 16 | >64 | 2 | ≤0.03 |
| Ceftolozane-tazobactam | >128 | >128 | >128 | 16 | 1 |
| Cefoperazone-sulbactam | >128 | >128 | >128 | ≤1 | ≤1 |
| Piperacillin-tazobactam | >256 | >256 | >256 | ≤2 | ≤2 |
| Ceftazidime-avibactam | 2 | >64 | 4 | 16 | 0.25 |
| Meropenem-vaborbactam | 2 | 1 | 2 | 0.06 | 0.03 |
| Cefepime-tazobactam | >64 | 64 | >64 | 0.125 | 0.125 |
| Cefepime-zidebactam | 2 | 1 | 2 | 0.125 | 0.125 |
| Ciprofloxacin | >8 | >8 | >8 | 4 | 0.25 |
| Levofloxacin | >16 | >16 | >16 | 4 | 0.25 |
| Trimethoprim-sulfamethoxazole | >32 | >32 | >32 | ≤0.25 | ≤0.25 |
| Tigecycline | 2 | 2 | 4 | 0.25 | 0.125 |
| Polymyxin B | 0.25 | 0.25 | 1 | 0.25 | 0.25 |

carbapenemases co-producing metallo-β-lactamases such as NDM, VIM, or IMP [4]; (2) single amino acid substitutions of $bla_{KPC}$, particularly at positions 164, 167, 169, and 179 within the Ω-loop of class A β-lactamases [5]; and (3) transposition of KPC with porin deficiency [6].

Herein, we report the first case of ceftazidime–avibactam resistance development during the treatment of CPE infections due to the change from KPC-2 to KPC-33 carbapenemase via the D179Y variant in China. Such mutations of plasmid-borne $bla_{KPC-2}$ can reduce the MICs of carbapenems (often restoring susceptibility to imipenem and low-level resistance to meropenem) [7, 8], because the mutations within the $bla_{KPC}$ Ω-loop (positions 165–179) enhance ceftazidime affinity and restrict avibactam binding [5]. Mutations such as R164S, L169P, D179Y, A177E, V240G, and T243M, and deletions in positions 167 and 168 are constantly emerging worldwide with the use of ceftazidime–avibactam, with D179Y as the most common amino acid substitution, especially in KPC [9–12]. Additionally, Ying Zhang et al. [13] reported that deficient OmpK35 and/or OmpK36 expression could be found in $bla_{KPC-2}$ positive *K. pneumoniae* (ST11 type) at Huashan Hospital. The frameshift mutation of the OmpK35-encoding gene found in our case indicates that OmpK35 porin may also be a minor contributor towards the drug resistance [13].

In the present case, ceftazidime–avibactam (2.5 g; administered intravenously every 8 h) seems not to work, though it has been previously confirmed to be effective against $bla_{KPC-2}$-positive *K. pneumoniae*, since the patient experienced persistent fever during the whole exposure period. The $bla_{KPC-33}$-positive strain emerged following the treatment of ceftazidime–avibactam for 16 days; this has been typically reported to occur after 10 to 19 days [7], and sometimes, after 33 days [14]. Subsequently, the $bla_{KPC-2}$-positive strain dominated again after the imipenem substitution therapy for ceftazidime–avibactam resistance. Thus, we speculated that $bla_{KPC-33}$ mutation via the D179Y variant of $bla_{KPC-2}$ was caused mainly by the selective pressure of ceftazidime–avibactam usage. Therefore, clinicians should be vigilant to ceftazidime–avibactam resistance when it is used for treating infections caused by $bla_{KPC-2}$-positive isolates; reintroduction of carbapenems is worthy of consideration on occasion.

Another potentially suitable antibiotic was meropenem–vaborbactam, which showed excellent in vitro antimicrobial activity and has been proven to be effective clinically [8]. Additionally, unlike ceftazidime–avibactam, no $bla_{KPC}$ mutations have been found to confer resistance to meropenem–vaborbactam [7].

Moreover, $bla_{KPC}$-positive *K. pneumoniae* with the ST258 clonal background are considered to represent the majority of ceftazidime–avibactam-resistant isolates [14]; however, the strain isolated from our patient was ST11-type *K. pneumoniae*. In China, 96.2% of ST11-type isolates produce KPC-2

carbapenemase, one of the most common carbapenemase types in *K. pneumoniae* (76.5%) [15]. More importantly, $bla_{KPC-2}$ positive *K. pneumoniae* ST11 strains can easily evolve pan-drug resistance through chromosomal mutations [16]. We speculate that mutant $bla_{KPC-33}$-positive *K. pneumoniae* ST11 strains may represent another main type of ceftazidime–avibactam resistant strains in China in the near future; thus, more attention may be required to monitor its prevalence.

Though detecting $bla_{KPC-33}$-positive strains is important for the choice of medication, it is easily mis-detected due to the inconspicuous characteristic of carbapenem resistance. NG-Test CARBA 5 and the RESIST-5, two popular direct rapid detection methods for $bla_{KPC-2}$-positive strains, show negative results for strains harboring $bla_{KPC-33}$. Routine molecular screening for $bla_{KPC}$ has also been advocated to facilitate its rapid detection [17].

Thus, ceftazidime–avibactam is one of the few antibiotics active against CPE. During its use, it is important to define optimal dosing strategies, insist on rigorous infection control practices, develop quick and efficient assays for detecting resistance, and further analyze resistance mechanisms. Once relevant cases are found, actions such as contact isolation and environmental cleaning may also be performed to avoid nosocomial outbreaks.

## Notes

***Author contributions.*** F. H. and R. Z. conceptualized and designed the overall study. Q. S., D. Y., R. H. and S. L. collected and analyzed the data. Y. G., Y. Z., S. W. and Y. Y. directed and managed the planning and execution of the project. F. H., Q. S. and D. Y. wrote and revised the paper. All authors reviewed and approved the final version of the manuscript.

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***Potential conflicts of interest***. All authors declare no conflict of interest.

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