Reduced Ceftazidime-Avibactam Susceptibility in KPC-Producing Klebsiella pneumoniae From Patients Without Ceftazidime-Avibactam Use History – A Multicenter Study in China

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KPC-producing Klebsiella pneumoniae (KPC-KP) is the most widely spread carbapenem-resistant Enterobacteriaceae (CRE) in China. Avibactam is a novel non-ß-lactam ß-lactamase inhibitor which is highly active against KPC. Recently, ceftazidime-avibactam (CAZ-AVI) was approved for clinical treatment in China. Here we conducted a retrospective study to examine the antimicrobial susceptibility of CAZ-AVI prior to its usage in China, and evaluated the potential to develop resistance in KPC-KP. CAZ-AVI MICs were tested in 347 KPC-KP isolates collected from patients with no prior treatment with this combination from six medical centers in China. Almost all isolates (n = 346; 99.7%) were CAZ-AVI-susceptible, with only 12 (3.5%) which showed reduced susceptibility (MIC ≥ 4/4 μg/ml) or resistance. The 12 isolates belong to ST11 and half of them carry virulence genes. In comparison to susceptible isolates, these isolates demonstrated higher blaKPC-2 copy numbers and expressions, and demonstrated higher frequency of developing CAZ-AVI resistance.

Keywords: KPC, Klebsiella pneumoniae, ceftazidime-avibactam, antimicrobial susceptibility, antimicrobial resistance

INTRODUCTION

With the widely use of carbapenem antibiotics, carbapenem-resistant Enterobacteriaceae (CRE) have been increasingly detected worldwide. The production of carbapenemases is the leading cause of carbapenem resistance in CRE. Klebsiella pneumoniae carbapenemase (KPC) is currently the most widely spread carbapenemase in the world, including China (Nordmann and Poirel, 2014),
while *K. pneumoniae* is the main clinical species producing KPC (Zhang et al., 2017). KPC-type β-lactamases could hydrolyze carbapenems and almost all β-lactam antibiotics, and traditional β-lactamase inhibitors have limited effects on KPC (Papp-Wallace et al., 2010). In addition, isolates producing KPC are commonly resistant to many other clinical agents (Pollett et al., 2014) due to the co-expression of several resistant determinants. Novel treatments for infections caused by KPC-producing *K. pneumoniae* (KPC-KP) are in urgent need.

Avibactam, a novel non-β-lactam β-lactamase inhibitor, had a spectrum of activity against β-lactamase of classes A (e.g., KPC), C (AmpC), and selected D (e.g., OXA-48) enzymes (van Duin and Bonomo, 2016). The combination of ceftazidime-avibactam (CAZ-AVI) has been approved in clinical treatment for KPC-producing *Enterobacteriaceae* by the United States Food and Drug Administration (FDA) in 2015 (FDA, 2015). In China, injectable CAZ-AVI (ZAVICEFTA®) was recently (May 2019) approved for the treatment of complicated intra-abdominal infections (cIAI) and hospital-acquired pneumonia (HAP), including ventilator associated pneumonia (VAP), caused by multidrug resistant Gram-negative bacteria.

In this study, we examined the CAZ-AVI susceptibility of KPC-KP from patients with no prior CAZ-AVI treatment history in China and evaluated the potential of isolates with reduced susceptibility to this combination to develop resistance through *in vitro* selection experiments.

**MATERIALS AND METHODS**

**Collection and Identification of Carbapenem-Resistant *K. pneumoniae***

A total of 616 unique clinical *K. pneumoniae* displaying carbapenem resistance which were defined as any isolate displaying imipenem and/or meropenem MIC values of >2 µg/ml based on CLSI guideline (Clinical and Laboratory Standards Institute, 2018) were collected from September 2016 to June 2018 from six tertiary hospitals in six different cities in China including Chengdu (Southwest China), Kunming (Southwest China), Guangzhou (South China), Yinchuan (Northwest China), Suzhou (Eastern China), and Beijing (Northern China). Species identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) method using the Bruker Daltonics MALDI Biotyper according to the instructions of manufacturer, and confirmed by 16S rRNA sequencing (Weisburg et al., 1991).

**Detection of Carbapenemases, ESBLs and Analysis of Porin Encoding Genes**

Polymerase chain reaction (PCR) was performed to detect the presence of carbapenemase-encoding genes (*bla*KPC, *bla*SIM, *bla*IMP, *bla*VIM, *bla*OXA-48) (Candan and Aksoz, 2015), the extended-spectrum beta-lactamases (ESBLs) genes (*bla*CTX-M, *bla*SHV, and *bla*TEM) (Bokaeian et al., 2015) and to amplify porin encoding genes (*omp*K35 and *omp*K36) (Clancy et al., 2013) in the carbapenem-resistant *K. pneumoniae*. The entire coding sequences for these genes were then amplified using previously published primers and conditions (Costa et al., 2009; Schechner et al., 2009) and subjected to Sanger sequencing.

**Determination of CAZ-AVI Minimal Inhibitory Concentrations**

The CAZ-AVI minimal inhibitory concentrations (MICs) were determined using the broth microdilution method recommended by CLSI (Clinical and Laboratory Standards Institute, 2018). *Escherichia coli* ATCC 25922 was used as quality control strain (Clinical and Laboratory Standards Institute, 2018). MICs were interpreted according to CLSI breakpoints (Clinical and Laboratory Standards Institute, 2018). A CAZ-AVI MIC of ≥4/4 µg/ml was used as the cut-off of reduced susceptibility to CAZ-AVI for this study (Shen et al., 2017).

**Antimicrobial Susceptibility Testing**

*In vitro* susceptibility of the isolates with reduced CAZ-AVI susceptibility against the commonly used clinical antimicrobials was evaluated by Phoenix 100 Automated Microbiology System and interpreted using CLSI (Clinical and Laboratory Standards Institute, 2018) guidelines or European Committee on Antimicrobial Susceptibility Testing (EUCAST) (for moxifloxacin and colistin). A total of 20 antibiotics belonging to 11 classes of antimicrobials were tested, including carbapenems (imipenem and meropenem), ureidopenicillins (piperacillin), β-lactam/β-lactamase inhibitor complexes (amoxicillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam), cephalosporins (cefazolin, cefotaxime, cefazidime, and ceftimeline), monobactams/β-lactams (aztreonam), aminoglycosides (gentamicin and amikacin), fluoroquinolones (ciprofloxacin, moxifloxacin and levofloxacin), folate metabolic pathway inhibitors (trimethoprim-sulfamethoxazole), chloramphenicol, colistin, and tetracycline.

**Collection of Clinical Information**

The clinical information including city, age range, isolation date, clinical department, sample, medical condition and outcome of the patients from whom the carbapenem-resistant *K. pneumoniae* was isolated was collected using EPIINFO software based on the medical records.

**Multilocus Sequence Typing (MLST)**

Multilocus sequence typing (MLST) was conducted to investigate the genetic relationships of the isolates with reduced CAZ-AVI susceptibility. PCR followed by Sanger sequencing was used to detect conserved housekeeping including *gapA, infB, mdh, pgI, phoE, rpoB*, and *tonB* (Diancourt et al., 2005). Allelic profiles and sequence types (STs) were determined using the *K. pneumoniae* MLST database¹.

**Pulsed-Field Gel Electrophoresis (PFGE)**

The clonal relatedness between the isolates with reduced CAZ-AVI susceptibility was investigated by pulsed-field gel

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¹https://bigsdb.pasteur.fr/klebsiella/klebsiella.html
electrophoresis (PFGE) with XbaI-digested DNA using a CHEF Mapper Power Module instrument (Bio-Rad, United States). Conditions of electrophoresis were as follows: voltage 6 V/cm, running time 20 h, temperature 14°C, and pulse times of 1–40 s. *Salmonella enterica* serotype Braenderup H9812 was used as size marker. The results were evaluated using GelJ v.2.0 analyzing software.

**Investigation of the Capsular Types and Virulence Genes**

Multiplex PCR-II analysis was applied to investigate the capsular types including K1, K2, KL64, KL47 (Yu et al., 2018), and multiplex PCR-III analysis was performed to detect four virulence types including K1, K2, KL64, KL47 (Yu et al., 2018), and *K. pneumoniae* rpoB as previously described (Kitchel et al., 2009). The statistical software used in this study was Prism 5 (Graph Pad Software). In addition, the *bla*KPC promoter regions from 12 isolates with reduced CAZ-AVI susceptibility and the 8 randomly selected susceptible isolates were amplified and sequenced using the primers (KPCpro-F, 5′-AACGTTGCTATCACGCAGCAT-3′ and KPCpro-R, 5′-CGAGTTTAGCAGAATGTTCC-3′), coverage the previously described promoter regions in *KPC*-KP isolates from China (Huang et al., 2019).

**In vitro Selection Testing**

All isolates underwent qRT-PCR detection, except for the CAZ-AVI (MIC 16/4 µg/ml) resistant isolate (*n* = 15), were subject to *in vitro* CAZ-AVI selective pressure testing, using a previously described method (Rodriguez-Villodres et al., 2020).
### TABLE 3 | Clinical information of the 12 patients connected with the isolates with reduced susceptibility to ceftazidime-avibactam.

| No. | City     | Age range (y) | Isolated date | Clinical department     | Source                | Antibiotic treatment before isolated | Carbapenem usage (days) | Outcome      |
|-----|----------|---------------|---------------|-------------------------|-----------------------|--------------------------------------|------------------------|--------------|
| 1758| Beijing  | 55–60         | Mar. 2017     | ICU                     | Respiratory tract     | Carbapenems                        | Meropenem (3)          | Deterioration|
| 1762| Beijing  | 30–35         | Mar. 2017     | ICU                     | Blood                 | Carbapenems                        | Meropenem (5) +          | Improve      |
| 1764| Beijing  | 35–40         | Mar. 2017     | ICU                     | Intra-abdominal       | Piperacillin-tazobactam, cephalosporin | –                      | Improve      |
| 1768| Beijing  | 45–50         | Mar. 2017     | ICU                     | Respiratory tract     | Carbapenems, piperacillin-tazobactam, tigecycline | Meropenem (11)        | Improve      |
| 2321| Beijing  | 35–40         | Apr. 2017     | General ward            | Blood                 | Carbapenems                        | Imipenem (5)           | Cure         |
| 2322| Beijing  | 35–40         | Apr. 2017     | General ward            | Respiratory tract     | Carbapenems                        | Imipenem (5)           | Cure         |
| 2477| Kunming  | 0–1           | Sep. 2017     | General ward            | Urinary tract         | Fluoroquinolone                    | –                      | Improve      |
| 2789| Kunming  | 0–1           | Oct. 2017     | General ward            | Urinary tract         | Piperacillin-tazobactam            | –                      | Improve      |
| 2827| Kunming  | 0–1           | Nov. 2017     | General ward            | Urinary tract         | Piperacillin-tazobactam            | –                      | Deterioration|
| 1706| Guangzhou| 60–65         | Feb. 2017     | ICU                     | Blood                 | Carbapenems, piperacillin-tazobactam, vancomycin | Meropenem (7) + Imipenem (1) | Improve      |
| 5152| Guangzhou| 55–60         | Sep. 2018     | Organ transplantation department | Blood                | Not clear                          | –                      | Not clear    |
| 3855| Suzhou   | 45–50         | Apr. 2018     | General ward            | Intra-abdominal       | Fluoroquinolone                    | –                      | Improve      |

*“–”, no carbapenems was used.*
### Table 4 | Molecular characteristics of the 12 KPC-KP with reduced susceptibility to ceftazidime-avibactam.

| No. | β-lactamases genes | Virulence genes | Sequence type | Capsular type | OmpK35 | OmpK36 |
|-----|---------------------|-----------------|---------------|---------------|--------|--------|
| 1758 | blaKPC-2, blaCTX-M-65, blaSHV-12 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 1762 | blaKPC-2, blaCTX-M-65, blaSHV-12 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 1764 | blaKPC-2, blaCTX-M-65, blaSHV-12 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 1768 | blaKPC-2, blaCTX-M-65 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 2321 | blaKPC-2, blaCTX-M-65, blaSHV-12 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 2322 | blaKPC-2, blaCTX-M-65, blaSHV-12 | rmpA, rmpA2, iutA, iroN | ST11 | KL64 | Stop codon | 134-135 GD |
| 2477 | blaKPC-2, blaCTX-M-14, blaSHV-12 | – | ST11 | KL47 | Stop codon | 134-135 GD |
| 2789 | blaKPC-2, blaCTX-M-14 | – | ST11 | KL47 | Stop codon | 134-135 GD |
| 2827 | blaKPC-2, blaCTX-M-14, blaSHV-12 | – | ST11 | KL47 | Stop codon | 134-135 GD |
| 1706 | blaKPC-2, blaCTX-M-65, blaSHV-12 | – | ST11 | KL64 | Stop codon | 134-135 GD |
| 5152 | blaKPC-2, blaCTX-M-65 | – | ST11 | KL47 | Stop codon | 134-135 GD |
| 3855 | blaKPC-2, blaCTX-M-65 | – | ST11 | KL64 | Stop codon | 134-135 GD |

“–”, negative for PCR detection.

### Figure 1 | PFGE cluster analysis of the 12 KPC-KP with reduced susceptibility to ceftazidime-avibactam. 1758, 1762, 1764, 1768, 2321, and 2322 were from Beijing sharing the same PFGE pattern. 2477, 2789, and 2827 were from Kunming, among which 2789 and 2827 were highly homologous (>90%). 1706 and 5152 were from Guangzhou. 3855 was from Suzhou.
RESULTS

Distribution of the KPC-KP
The 616 non-duplicate clinical K. pneumoniae collected from September 2016 to June 2018 were from Chengdu (226, 36.7%, Southwest China), Kunming (148, 24.0%, Southwest China), Guangzhou (90, 14.6%, Southern China), Yinchuan (62, 10.1%, Northwest China), Suzhou (47, 7.6%, Eastern China), and Beijing (43, 7.0%, Northern China). In this collection 347 (56.3%) K. pneumoniae contained blaKPC, without coexistence of metallo-β-lactamases genes, the majority of which were from Kunming (119, 34.3%) followed by Chengdu (97, 28.0%), Guangzhou (49, 14.1%), Beijing (34, 9.8%), Suzhou (25, 7.2%), and Yinchuan (23, 6.6%) (Table 1).

Antimicrobial Susceptibility of KPC-KP
Minimal inhibitory concentrations of CAZ-AVI inhibiting KPC-KP ranged from ≤0.125/4 to 16/4 µg/ml, with only one strain which was resistant (16/4 µg/ml) according to the CLSI breakpoint (Clinical and Laboratory Standards Institute, 2018). The MIC50 was 1/4 µg/ml and MIC90 was 2/4 µg/ml (Table 1). We then used CAZ-AVI MIC of ≥4/4 µg/ml as the cut-off of reduced susceptibility to CAZ-AVI for this study (Shen et al., 2017). A total of 12 KPC-KP were found to have a CAZ-AVI MIC value ≥4/4 µg/ml with high level resistance to carbapenems (meropenem MICS ≥ 256 µg/ml) (Table 1).

These 12 isolates showed resistance to nearly all tested antimicrobials except trimethoprim-sulfamethoxazole (41.7%), chloramphenicol (16.7%), colistin (0%), and tetracycline (41.7%) (Table 2).

Clinical Information
The 12 isolates were obtained from distinct patients aged between 0 and 65 years old. They were from Beijing (n = 6), Kunming (n = 3), Guangzhou (n = 2), and Suzhou (n = 1). Four out of six Beijing isolates were collected from patients in ICU wards within the same month. In addition, most of these patients received empirical antibiotic treatments before these isolation of strains. Among them, carbapenems were the most commonly used antibiotic, followed by piperacillin-tazobactam (Table 3).

Detection of ESBL and Virulence Genes and Capsular Genotyping
These 12 isolates contain ESBLs genes including blaCTX−M−65 (n = 9), blaCTX−M−14 (n = 3) and blaSHV−12 (n = 8). In addition, the results of multiplex PCR showed that 6 isolates from Beijing were positive for the four virulence genes, namely rmpA, rmpA2, iroN, and intA. Eight isolates belonged to capsular type KL64 and the other 4 isolates were from KL47 (Table 4).

MLST Sequence Types and PFGE Patterns
Multilocus sequence typing results showed that all 12 isolates belonged to ST11. Furthermore, these 12 isolates were investigated by PFGE. As shown in Figure 1, the 6 isolates from Beijing shared the same PFGE pattern. In addition, 2 of the 3 isolates from Kunming were highly homologous (>90%, dice similarity coefficient). The other isolates demonstrated different pulsotypes.

Outer Membrane Porin Gene Sequence Analysis
Sequencing of the outer membrane porin genes ompK35 and ompK36 showed that all 12 isolates contain a mutant OmpK35, with a premature stop codon at amino acid position 63, as well as a mutant OmpK36, due to the glycine and aspartic acid duplication at amino acid 134 (134–135 GD insertion). However, the OmpK35 and OmpK36 gene sequences of 8 randomly selected susceptible K. pneumoniae ST11 isolates showed the same genotypes (OmpK35 stop codon and OmpK36 134–135 GD insertion) as those of the 12 isolates with reduced CAZ/AVI susceptibility.

Mechanism of Reduced CAZ-AVI Susceptibility
Polymerase chain reaction detection and Sanger sequencing showed that the 12 isolates all harbored wild-type blaKPC−2. Examination of the blaKPC−2 promoter regions failed to identify any mutations in comparison to the sequences from the susceptible strains. The results of qRT-PCR showed that the relative blaKPC−2 copy numbers in the reduced susceptibility group were significantly higher than those in the susceptibility group (2.6-fold, P = 0.0004) (Figure 2A). In addition, the relative expressions of blaKPC−2 in the reduced susceptibility group were 3.9-fold higher than those in the susceptibility group (P = 0.0034) (Figure 2B).

In vitro Selection Testing
The in vitro selection experiments showed that 4 of the 7 isolates with reduced susceptibility developed resistance to CAZ-AVI at the selection concentration of 2/4 µg/ml, and all 7 isolates developed resistance to CAZ-AVI with MICs ranging from 32/4 to 256/4 µg/ml when the selection concentration reached 4/4 µg/ml. By contrast, no isolates were developed to be resistance to CAZ-AVI in the susceptibility group, and all eight isolates stopped growing when the selection concentration reached 8/4 µg/ml.

DISCUSSION
At present time, KPCs are the most common carbapenemases identified worldwide especially in K. pneumoniae from China (Nordmann and Poirel, 2014). Due to the limited therapies, the detection rate of KPC-KP in China demonstrated a continuous upward trend (Hu et al., 2016). In this study, CAZ-AVI showed potent in vitro activities against KPC-KP, which agreed with previous studies (de Jonge et al., 2016; Spiliopoulou et al., 2020). However, ~3% isolates displayed reduced susceptibility to CAZ-AVI (MIC ≥ 4/4 µg/ml) despite they were obtained from patients without previous CAZ-AVI treatment history. Notably, these isolates also showed high level resistance to carbapenems.
In 2018, a fatal outbreak caused by ST11 KPC-KP with acquisition of a pLVPK-like virulence plasmid that increased the virulence of these isolates was reported (Gu et al., 2018). In our study, half of the isolates with reduced CAZ-AVI susceptibility contained several known virulence genes, suggesting that these isolates may have increased virulence, which should be closely monitored.

Since the previously reported gene mutations (e.g., D179Y) associated to CAZ-AVI resistance (Giddins et al., 2018) weren’t found in the \( \text{bla}_{\text{KPC}-2} \) genes, this study suggested that mechanisms other than \( \text{bla}_{\text{KPC}-2} \) gene mutation were underlying the reduced CAZ-AVI susceptibility among these isolates. OmpK35/36 defects had previously been reported to lead to reduced susceptibility or resistance to CAZ-AVI in KPC-KP (Nelson et al., 2017). In this study, our results showed 12 isolates with reduced CAZ-AVI susceptibility contained OmpK35 and OmpK36 gene mutations, however, the same gene mutations were also found in the susceptible strains, which suggested that OmpK35/36 defects may only partially contribute to the reduced CAZ-AVI susceptibility among those 12 strains, while additional mechanisms may be involved. Our results demonstrated that the \( \text{bla}_{\text{KPC}-2} \) copy numbers and expressions in the reduced susceptibility group were significantly higher than those in the susceptible group. We therefore suspected that the reduced CAZ-AVI susceptibility in these strains was likely due to the higher \( \text{bla}_{\text{KPC}-2} \) copy numbers and gene expressions, in combination to the OmpK35/36 defects. The results were consistent with some previously published studies (Shen et al., 2017; Zhang et al., 2020). The higher copy numbers of \( \text{bla}_{\text{KPC}-2} \) may potentially result from the high copy numbers of the plasmids carrying \( \text{bla}_{\text{KPC}} \) (Roth et al., 2011) and/or a duplication of mobile genetic elements associated with \( \text{bla}_{\text{KPC}} \) (Coppi et al., 2020). Since the examination of the \( \text{bla}_{\text{KPC}-2} \) promoter regions failed to identify any mutations, the higher \( \text{bla}_{\text{KPC}} \) gene expression may potentially be affected by the higher copy numbers of \( \text{bla}_{\text{KPC}} \) or other gene regulatory mechanisms. Further studies, including whole genome sequences, are needed to explore the molecular mechanisms underlying the higher \( \text{bla}_{\text{KPC}-2} \) copy numbers and gene expressions among those strains.

Shields et al. (2017) has reported on the acquisition of CAZ-AVI resistance among ST258 KPC-KP during treatment in the United States. After that, (Raisanen et al., 2019) isolated a ST39 KPC-KP that was resistant to CAZ-AVI after this combination treatment. In China, ST11 KPC-KP is commonly prevalent (Chen et al., 2014; Zhang et al., 2017). In this study, the ST11 KPC-KP with reduced susceptibility were more prone to develop CAZ-AVI resistance compared to susceptible isolates under the pressure of CAZ-AVI exposing.

Taken together, our study demonstrated that CAZ-AVI has potent \textit{in vitro} activities against KPC-KP in China and highlighted the clinical significance of the isolates with reduced susceptibility to CAZ-AVI isolated from patients without previous CAZ-AVI treatment history. Our results suggested that the optimal clinical usage of CAZ-AVI should be guided by the \textit{in vitro} susceptibility results in order to prevent selection resistance.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.
ETHICS STATEMENT
This study was approved by the institutional review board (IRB) of The Second Affiliated Hospital of Soochow University. The clinical isolates were retrospectively collected, and patient data were not included in this study, therefore the need for written informed consent was waived by the IRB.

AUTHOR CONTRIBUTIONS
XC, BS, and XZ contributed to conducting the study, data analysis, and manuscript preparation. FQ, WJ, BH, HY, and Y-WT analyzed the data and reviewed the manuscript. LC and HD contributed to study design, data analysis, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: Y-WT was employed by Cepheid, Shanghai, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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