Emergence of Hypervirulent Ceftazidime/Avibactam-Resistant *Klebsiella pneumoniae* Isolates in a Chinese Tertiary Hospital

**Introduction:** Carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) is increasingly reported worldwide, but ceftazidime/avibactam (CAZ/AVI)-resistant hvKP isolates have rarely been observed. We attempted to characterize them in clinical CRKP isolates collected from a university hospital in China from March 2016 to March 2018.

**Methods:** All isolates were analyzed by antimicrobial susceptibility testing, molecular detection of antibiotic resistance determinants, multilocus sequence typing (MLST), SDS-PAGE, and pulsed-field gel electrophoresis (PFGE). The pLVPK-related genetic loci (*rmpA2, terW, iutA*, and *silS*) were screened in all CAZ/AVI-resistant CRKP isolates for the presence of virulence plasmids by PCR. Capsule typing, serum killing assay, *Galleria mellonella* lethality experiments, and mouse lethality assay were conducted to identify CAZ/AVI-resistant hvKP among isolates that carried all four virulence genes.

**Results:** A total of 232 CRKP isolates were collected. Overall, CAZ/AVI-resistance was found in 8.2% (19/232) CRKP isolates isolated from patients with no history of previous CAZ/AVI-based treatment. Among these, 63.2% (12/19) were metallo-β-lactamase-producing *K. pneumoniae* (MBL-KP), 52.6% (10/19) were *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-KP), and 26.3% (5/19) produced both MBL and KPC. The presence of carbapenemase promoted a very high increase in CAZ/AVI minimum inhibitory concentration only when *ompK35* and *ompK36* were absent. Alarming, nine isolates had all four virulence genes for the presence of virulence plasmids. All nine isolates were considered to be CAZ/AVI-resistant hvKP according to the *G. mellonella* infection model and mouse lethality assay, with ST23 being the most common type (55.6%, 5/9).

**Conclusion:** The newly emerged hypervirulent CAZ/AVI-resistant KP strain might cause a serious threat to public health, suggesting an urgent need for enhanced clinical awareness and epidemiologic surveillance.

**Keywords:** ceftazidime/avibactam-resistant, hypervirulent, *Klebsiella pneumoniae*, *ompK35*
need for new drugs with anti-CRKp activity has finally been addressed by the recent introduction of novel β-lactam/β-lactamase inhibitor combinations, among which ceftazidime-avibactam (CZA/AVI) was the first to be released for clinical use.3

Ceftazidime-avibactam is a new type of β-lactam/β-lactamase inhibitor combination that is effective against Enterobacteriaceae producing KPCs, OXA-48, carbapenemases, extended-spectrum β-lactamases, and AmpC β-lactamases, but it is not effective against those producing metallo-β-lactamases.4 A previous study has shown that CZA/AVI provides a valuable alternative strategy against CR-hvKP, including the KPC-2-producing ST11 hvKP isolates, which are increasingly isolated in China.5 Although CZA/AVI-based therapies have shown initial optimistic clinical efficacy in treating severe KPC-Kp infections, recent reports of development of drug resistance have been cause for concern.6,7 Previous studies showed that CZA/AVI resistance was associated with different mutations in the KPC enzyme or overexpression of the blaKPC gene associated with non-functional porin.8,9 However, to date, clinical isolates of CR-hvKP resistant to CZA/AVI have been rarely found. Therefore, this study aims to investigate the patterns of hypervirulence in CZA/AVI-resistant CRKP and further analyze the mechanisms of resistance.

Materials and Methods
Bacterial Isolates and Antimicrobial Susceptibility Tests
Between March 2016 and March 2018, 232 CR-KP isolates were isolated from patients hospitalized in a large tertiary hospital in Southern China. Antimicrobial susceptibility testing was performed using the VITEK II system (bioMérieux, Balmes-Les-Grottes, France) and confirmed by microdilution method. Results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines.9 All isolates were characterized as resistant to imipenem or meropenem and were determined with phenotypic screening for carbapenemase production. The breakpoint of CZA/AVI was based on the interpretative criteria according to K-B method of CLSI. Results of tigecycline and colistin were interpreted according to the breakpoint approved by the EUCAST.10 The reference strains—K. pneumoniae ATCC 700603 and Escherichia coli ATCC 25922—were used for quality control of antimicrobial susceptibility testing.

Capsular Serotyping, Resistance Genes, and pLVK-Related Genes Detection
Polymerase chain reaction (PCR) was used to detect resistance genes related to carbapenemases (GES, KPC, NMC, SME, IMP, VIM, GIM, SPM, SIM, NDM, and OXA); β-lactamase (SHV, TEM, CTX-M, VEB, CMY, and DH1); and fenestra protein genes (ompK35 and ompK36) of the bacterial isolates. The eight capsular serotyping genes (K1, K2, K5, K20, K54, K37, K47, and K64) and pLVK-related genetic loci (rmpA2, terW, intA, silS) were also determined by PCR and sequencing as previously described.11-16

Outer Membrane Protein Isolation and SDS-PAGE
Outer membrane proteins (OMPs) were isolated according to the rapid procedure of Carlone et al and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.17 The reference strains K. pneumoniae ATCC 700603 and K. pneumoniae NUTH-K2044 were also used as quality control.

G. mellonella Infection Model
All the clinical K. pneumoniae isolates were grown to the late exponential phase in MH broth; the isolates were then collected by centrifugation and suspended in saline. Ten larvae weighing between 250 and 350 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) were used for the assessment of the virulence level of each strain. The larvae were inoculated by injecting 1×10^6 CFU of clinical K. pneumoniae isolates per 10 microliters aliquot into the hemocoel via the rear left proleg using a 10-μL Hamilton animal syringe.15 Ten larvae were also injected with 1×10^6 CFU ATCC700603 and 1×10^6 CFU NTUH-K2044, respectively, as the controls. Larvae were incubated at 37°C and observed every 24 h for 5 days to monitor mortality. The average survival time of each group of infected larvae was recorded.

Serum Killing Assay
Serum killing assay was performed as described previously.18 Briefly, prior to the assay, serum separated from 10 healthy individuals’ blood was stored at ~80°C. A 10^6 CFU of bacteria-containing inoculum prepared from the mid-log phase was reacted with 75% pooled human sera. The final mixture was incubated at 37°C, and we obtained viable counts at 0, 1, and 3 h,
respectively. The response to serum killing in terms of viable counts was scored using six grades classified as serum sensitive (grade 1 or 2), intermittently sensitive (grade 3 or 4) or serum resistant (grade 5 or 6).

S1-PFGE and Southern Blot
After PCR screening of pLVPK-related genetic loci, S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) and southern blotting hybridization were performed to determine the location of virulence genes. Briefly, total DNA was embedded in agarose gel plugs. The plugs were digested with S1 nuclease (TaKaRa) at 37°C for 30 min and then separated by electrophoresis. Labeling of the probe rpmA2 and hybridization were performed with the DIG-High Prime DNA Labeling and Detection Starter Kit II, according to the manufacturer’s instructions (Roche, Basel, Switzerland). 19

Mouse Lethality Assay
Determination of the virulence of KP in mouse lethality tests and the medium lethal dose (LD50, expressed as colony-forming units) was performed as previously described. 20 In brief, a graded dose of 10¹ to 10⁶ CFU of each strain in 10-fold serial dilutions in 0.1 mL of normal saline was injected intraperitoneally into mice (4 mice for each dose of inoculum). All inoculated mice were recorded daily for survival. Interpretation of virulence was referred to reference. 21

Multilocus Sequence Typing (MLST) and PFGE
Seven conserved housekeeping genes—gapA, infB, mdh, pgI, phoE, rpoB, and tonB—were used to perform MLST (http://biggsb.pasteur.fr/klebsiella/klebsiella.html). Clonal relatedness among CAZ/AVI-resistant K. pneumoniae isolates was established using XbaI - PFGE (TaKaRa). DNA fragments were separated with a CHEF DR III apparatus (Bio-Rad, Richmond, CA, USA). The molecular marker was Salmonella serotype Braenderup strain H9812. The isolates sharing >80% similarity were defined as the same PFGE cluster. 22

Ethics Statement
The study has been evaluated by the Ethics Committee of the First Affiliated Hospital of Nanchang University (ethical number: 2019130). Patients involved in the study were anonymized, and the need for informed consent was waived because of the retrospective nature of the study.

Results
Prevalence of Carbapenemase Genes and β-Lactamase Genes Among CAZ/AVI-Resistant K. pneumoniae Clinical Isolates
A total of 232 carbapenem-resistant K. pneumoniae clinical isolates were collected from March 2016 to March 2018 in our hospital. Among these, 19 CAZ/AVI-resistant K. pneumoniae clinical isolates were selected from clinical specimens including 10 sputum, 4 blood, 3 urine, and 2 pus. As shown in Supplementary Table 1, almost all the CAZ/AVI-resistant K. pneumoniae clinical isolates were resistant to 20 antibiotics commonly used in clinical treatment, except for some isolates that were sensitive to tigecycline, polymyxin, and amikacin. Hence, these CAZ/AVI-resistant K. pneumoniae clinical isolates, particularly CAZ/AVI-resistant hvKP isolates, understandably pose massive challenges for clinical treatment. As shown in Table 1, almost all of the CAZ/AVI-resistant K. pneumoniae clinical isolates were found to carry at least one carbapenemase gene or extended-spectrum β-lactamase gene. Interestingly, among these CAZ/AVI-resistant K. pneumoniae clinical isolates, NDM-1/NDM-5 were more likely clustered in hvKP in our hospital.

Virulence Assessment of CAZ/AVI-Resistant K. pneumoniae Clinical Isolates
In this scenario, capsular serotyping showed there were 10 K46 isolates, five K1 isolates, two K2 isolates, one K14 isolate, and one K15 isolate among CAZ/AVI-resistant K. pneumoniae clinical isolates. PCR analysis of pLVPK-related genetic loci revealed there were nine CAZ/AVI-resistant K. pneumoniae isolates that may carry the pLVPK-like virulence plasmid. These results were verified by PFGE and Southern blotting. As shown in Supplemental Figure 1, nine (47.4%) CAZ/AVI-resistant hvKP clinical isolates were identified among 19 CAZ/AVI-resistant K. pneumoniae clinical isolates. Moreover, the serum killing assay, G. mellonella infection model, and mouse lethality assay also proved that the nine CAZ/AVI-resistant hvKP clinical isolates were hypervirulent.
Table 1 Virulence Phenotype of CAZ/AVI-Resistant K. pneumoniae Isolates and Drug-Resistant Genes

| Isolates | Specimen | Extended Spectrum β-Lactamases and Carbapenemase Genes | Outer Membrane Protein Genes | pLVPK-Related Loci | Serum Killing Assay | Galleria mellonella Infection Model | LDS0 (CFU) |
|----------|----------|--------------------------------------------------------|-----------------------------|--------------------|-------------------|---------------------------------|---------|
| A18      | Sputum   | KPC-2, TEM-1                                           | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| I5       | Blood    | KPC-2, DHA-1, TEM-1, CTX-M-15                          |                             | rpmA2, terW, iutA, silS | Grade 5            | <24h                            | 5.4×10² |
| XY5      | Sputum   | KPC-2, SHV-12, TEM-1, CTX-M-15                         | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| FZ1      | Sputum   | -                                                      | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁴  |
| 21       | Blood    | KPC-2, TEM-1, CTX-M-15                                  | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| GZ20     | Sputum   | KPC-2, DHA-1, SHV-12, TEM-1, CTX-M-15                   |                             | rpmA2, terW, iutA, silS | Grade 5            | <24h                            | 1.8×10⁵ |
| 356      | Sputum   | NDM-1, DHA-1, SHV-12, TEM-1                             | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| A22      | Sputum   | NDM-1, TEM-1                                           | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| GZ19     | Urine    | NDM-1, DHA-1                                          | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |
| JDZ25    | Sputum   | NDM-1, KPC-2, DHA-1, SHV-12, TEM-1, CTX-M-15           | ompK35, ompK36              |                    | Grade 1            | 120h                            | >1×10⁶  |

Table 1 shows the virulence phenotype of CAZ/AVI-resistant K. pneumoniae isolates and drug-resistant genes. SDS-PAGE of Outer Membrane Proteins

The outer membrane proteins of 19 CAZ/AVI-resistant K. pneumoniae clinical isolates were detected by SDS-PAGE. Two ST11 isolates lacked outer membrane proteins, as shown in Figure 1. PCR analysis of the outer membrane protein genes also revealed absence of the OmpK35 and OmpK36 genes. Therefore, unlike the other seven CAZ/AVI-resistant hvKP isolates carrying the NDM gene, these two ST11 hvKP isolates were resistant to CAZ/AVI because of loss of the outer membrane proteins.
Molecular Characteristics

Among the 19 CAZ/AVI-resistant *K. pneumoniae* isolates, five STs were identified, including ST11 (10 K64 isolates), ST23 (five K1 isolates), ST86 (two K2 isolates), ST345 (one K14 isolate), and ST4065 (one K25 isolate). The PFGE patterns were assigned to four clusters based on >80% pattern similarity, comprising seven ST11 isolates, five ST23 isolates, three ST11 isolates, and two ST86 isolates (Figure 2). The remaining two isolates (ST14 and ST25) had different pulsotypes. The PFGE and MLST results suggested that CAZ/AVI-resistant hvKP isolates mainly clustered in K1 ST23 isolates and K2 ST86 isolates.

Discussion

The use of CAZ/AVI against carbapenem-resistant *K. pneumoniae* strains has been shown to be very effective to provide a higher clinical cure rate and survival rate in China, the United States, and other European countries. However, increased reports of CAZ/AVI resistance in carbapenem-resistant *K. pneumoniae*, regardless of prior exposure, is a matter of concern. A national research about CAZ/AVI resistance in China revealed that MBL production, *blaKPC-2* point mutation and high KPC expression played an important role in CAZ/AVI resistance. Nevertheless, reports of CR-hvKP with CAZ/AVI resistance have been rare.

Here, we described the emergence of 19 CAZ/AVI-resistant carbapenem-resistant *K. pneumoniae* isolates including nine CR-hvKP isolates isolated from patients with no history of previous antimicrobial exposure to CAZ/AVI treatment. Our results showed that CAZ/AVI resistance was mainly associated with the presence of metallo-β-lactamases or deficiency of OmpK35/36 porins. Although previous studies have demonstrated that the mechanisms responsible for CAZ/AVI resistance included specific mutations within the *blaKPC* gene, PCR analysis of the *KPC* gene in this study showed no mutations in the *KPC-3* gene. As described in a previous study, CAZ/AVI resistance and KPC-3 amino acid substitutions only occurred at low frequencies (~10⁻⁹) in

Figure 1 SDS-PAGE of outer membrane proteins of 19 clinical CAZ/AVI-resistant *K. pneumoniae* isolates. Note: the dotted rectangle reveals the absence of OmpK35 and OmpK36.
K. pneumoniae during in vitro selection. Moreover, due to the limited use of CAZ and AVI in our hospital, the probability of genetic mutations was further reduced.

SDS-PAGE of the outer membrane protein in this study revealed that only two ST11 isolates that carried the pLVPK-like virulence plasmids lacked the outer membrane proteins. Therefore, the loss of porin OmpK35/36 was not a primary and direct cause of resistance to CAZ/AVI in this study. Per previous studies, the OmpK35 and OmpK36 porins were not thought to be the initial pathway for AVI into K. pneumoniae, rather the combination of this with increased KPC expression or production of DHA-1 β-lactamases could likely have led to the CAZ/AVI resistance phenotype.

Importantly, nine (47.4%) CAZ/AVI-resistant hvKP clinical isolates carrying pLVPK-like virulence plasmids were identified in this study. The serum killing assay and G. mellonella infection model and mouse lethality assay also proved that the nine CAZ/AVI-resistant hvKP clinical isolates were more virulent than the remaining CAZ/AVI-resistant clinical isolates. Although Yu et al. found that CAZ/AVI remained highly active against KPC-2-producing ST11 hvKp isolates, two CAZ/AVI-resistant ST11 CR-hvKP clinical isolates had first emerged in our hospital because of porin OmpK35/36 loss and DHA-1 β-lactamases production.

The PFGE and MLST results of the 19 CAZ/AVI-resistant K. pneumoniae isolates revealed that the CAZ/AVI-resistant hvKP in this study were the ST23 (K1)/ST86 (K2) isolates carrying the NDM gene and the ST11 KPC-hvKP isolates that lacked OmpK35/36 porins and DHA-1 β-lactamases production. Both these types of CAZ/AVI-resistant hvKP isolates need further monitoring.

Our study has some limitations, including its retrospective nature and a relatively small study population. Therefore, there may be selection bias, which limits the applicability and generalizability of our results to other areas. Although we did not discuss efflux pump mechanisms.
of CAZ/AVI resistance, to our best knowledge, this is the first description of CAZ/AVI resistance in carbapenem-resistant hypervirulent Klebsiella pneumoniae.

**Conclusion**
The major mechanisms of CAZ/AVI resistance among carbapenem-resistant Klebsiella pneumoniae in our hospital were metallo-β-lactamases production and loss of porin OmpK35/36 along with DHA-1 β-lactamases production. Effective infection control measures are urgently needed to prevent the emerging hypervirulent CAZ/AVI-resistant hvKP strains from becoming an epidemic in the future.

**Abbreviations**
CR-hvKP, carbapenem-resistant hypervirulent Klebsiella pneumoniae; CAZ/AVI, ceftazidime-avibactam; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; MBL-KP, metallo-β-lactamase-producing K. pneumoniae; KPC-KP, Klebsiella pneumoniae carbapenemase (KPC)-producing K. pneumoniae; G. mellonella, Galleria mellonella; CR-KP, carbapenem-resistant Klebsiella pneumoniae; S1-PFGE, S1 nuclease-pulsed-field gel electrophoresis; CLSI, the Clinical and Laboratory Standards Institute; PCR, polymerase chain reaction; OMPs: outer membrane proteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LD50, the medium lethal dose.

**Author Contributions**
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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**Disclosure**
The authors report no conflicts of interest in this work.

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