

# Prevalence, Antimicrobial Susceptibility, and Molecular Characteristics of Ceftazidime-Avibactam-Resistant *Klebsiella pneumoniae* in a Chinese Hospital

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**Background:** The mechanisms underlying ceftazidime-avibactam resistance in carbapenem-resistant *Klebsiella pneumoniae* (CRKP<sup>CZA-R</sup>) remain to be elucidated.

**Methods:** CRKP<sup>CZA-R</sup> isolates were screened from non-repetitive CRKP isolates at our hospital from January 1, 2018 to October 30, 2021. The antimicrobial susceptibility and molecular characteristics of CRKP<sup>CZA-R</sup> were analyzed by broth microdilution method and next-generation sequencing, respectively.

**Results:** In total, 67 of 623 CRKP isolates (10.8%) were identified as CRKP<sup>CZA-R</sup>. The susceptibility rates of CRKP<sup>CZA-R</sup> to polymyxin B, tigecycline, aztreonam-avibactam, and cefiderocol were 97.0% (65/67), 83.6% (56/67), 100.0% (67/67), and 94.0% (63/67), respectively. The most prevalent resistance gene was *bla*<sub>NDM-1</sub> (44.8%, 30/67), followed by *bla*<sub>IMP-4</sub> (9.0%, 6/67), *bla*<sub>NDM-5</sub> (7.5%, 5/67), and *bla*<sub>NDM-4</sub> (1.5%, 1/67). Furthermore, 37.3% (25/67) of the CRKP<sup>CZA-R</sup> isolates co-harbored more than two carbapenemase-encoding genes, mainly *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub> (31.3%, 21/67). The enzyme inhibitor enhancement method detected carbapenemase activity with high sensitivity, except for isolates carrying two or more carbapenemases. Notably, 21 KL64-ST11 CRKP<sup>CZA-R</sup> isolates presented *bla*<sub>NDM-1</sub>, *bla*<sub>KPC-2</sub>, and *ompk36* deletion, and 17 co-harbored two or more high virulence gene markers. Patients infected with these 21 isolates were older and experienced more serious illness compared to those infected with other drug-resistant isolates.

**Conclusion:** The detection rate of CRKP<sup>CZA-R</sup> was relatively high due to the metallo-β-lactamase-producing isolates. Although enzyme inhibitor enhancement method can detect carbapenemases with high sensitivity and specificity, and provide an important reference for drug selection, it is not as effective for isolates carrying two or more carbapenemases. Patients infected with CRKP<sup>CZA-R</sup> co-harboring both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> should be closely monitored.

**Plain Language Summary:** The resistance mechanisms of CRKP<sup>CZA-R</sup> remain to be uncovered. In our study, the detection rate of CRKP<sup>CZA-R</sup> was relatively high due to the metallo-β-lactamase-producing isolates. The sensitivity of CRKP<sup>CZA-R</sup> to antibacterial agents was evaluated in vitro, and revealed high susceptibility to aztreonam-avibactam, cefiderocol, polymyxin B, and tigecycline. The enzyme inhibitor enhancement method plays an important role in guiding the selection of clinical antibiotics due to its high sensitivity and specificity. Specifically, when the inhibition zone diameter of K-B method CZA is 20-22 mm, CZA can be used alone if it produces serine enzyme, but not if it produces metalloenzyme. Patients infected with CRKP<sup>CZA-R</sup> harboring both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> were older and experienced more serious illness compared to those infected with other drug-resistant isolates, and should therefore be monitored more closely.

**Keywords:** carbapenem-resistant *Klebsiella pneumoniae*, ceftazidime-avibactam, resistance mechanism, homology



## Introduction

The global prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has increased in recent years, resulting in big challenges in the treatment of bacterial infections.<sup>1–3</sup> On May 17, 2024, CRKP was listed as part of the *critical group* in the latest priority list of bacterial pathogens issued by World Health Organization (WHO).<sup>4</sup>

Ceftazidime-avibactam (CZA) is a novel  $\beta$ -lactamase inhibitor consisting of ceftazidime (CAZ) and avibactam (AVI). With the increasing prevalence of infections caused by bacterial isolates resistant to traditional antibiotics, there is an urgent need to design more effective therapeutic options.<sup>5</sup> CZA is effective against a range of pathogens, including Enterobacteriales and *Pseudomonas aeruginosa*,<sup>6,7</sup> and has been recommended by the Infectious Diseases Society of America (IDSA) for treating infections caused by CRKP. However, the widespread use of CZA has led to the emergence of CZA-resistant CRKP (CRKP<sup>CZA-R</sup>) isolates in several countries, and the resistance rate in some regions is as high as 21%.<sup>8,9</sup>

The most common mechanism driving CZA resistance is the production of metallo- $\beta$ -lactamases (MBLs), including New Delhi MBL, imipenemase, and Verona integron-encoded MBL. As AVI cannot bind to MBLs and block the hydrolysis of CAZ, MBL-producing isolates are naturally resistant to CZA.<sup>10</sup> Nevertheless, AVI can protect CAZ from serinases. Although CZA has been effective in the treatment of infections caused by carbapenemase-producing CRKP, studies show that KPC-producing isolates can develop resistance to CZA. Selective mutations in one or more amino acids in the  $\Omega$  loop of KPC-2 and KPC-3 can render them impervious to the inhibitory action of AVI, resulting in novel CZA-resistant CRKP variants.<sup>11</sup> Novel KPC subtypes, including KPC-33, KPC-121, and KPC-125, have been continuously reported among CZA-resistant CRKP isolates.<sup>12–14</sup> The CRKP isolates producing KPC-31 and KPC-33 variants, which are the result of D179Y and H274Y mutations, respectively, are currently the most common isolates isolated in clinics worldwide.<sup>15</sup> Furthermore, increased expression of the *bla*<sub>KPC</sub> gene,<sup>16</sup> mutations in LamB (outer membrane maltoprotein), and high expression of *bla*<sub>SHV-12</sub> have also been associated with CZA resistance.<sup>17,18</sup>

In this study, we screened CRKP<sup>CZA-R</sup> isolates among non-repetitive clinical isolates of CRKP collected at our hospital from January 1, 2018 to October 30, 2021. The drug resistance genes, virulence-related genes, and sequence types (STs) of these isolates, and the clinical data of infected patients were analyzed to clarify the epidemiological characteristics of CRKP<sup>CZA-R</sup>, and provide an experimental basis for treating infections caused by CZA-resistant isolates.

## Materials and Methods

### Bacterial Isolates

In order to clarify the epidemiological characteristics of CRKP<sup>CZA-R</sup>, all non-repetitive clinical CRKP isolates were collected at our hospital between January 1, 2018 and October 30, 2021, and re-identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Zhongyuan Huiji Biotechnology Co. Ltd, China) using *Escherichia coli* ATCC 25922 as the quality-control isolate. Patient data were also collected, including age, sex, ward, clinical diagnosis, underlying diseases, complications, prior invasive procedures, clinical outcomes, and antibiotic usage before and after isolation.

The quality control isolates (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853) were purchased from National Center for Clinical Laboratories (Beijing, China).

### Antimicrobial Susceptibility Testing

The K-B method was used to screen CZA-resistant CRKP isolates (30/20- $\mu$ g Disk, Oxoid). For isolates with inhibition zone diameters of 20–22 mm, the respective minimum inhibitory concentrations (MICs) of CZA were determined by the microdilution broth method as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI).<sup>19</sup> The *K. pneumoniae* ATCC 700603 isolate was also included in the drug sensitivity test for quality control.

The MICs of meropenem (MEM), imipenem (IPM), polymyxin B (POL), tigecycline (TGC), fosfomicin (FOS), aztreonam-avibactam (AZA), and cefiderocol against CRKP<sup>CZA-R</sup> were determined by the broth microdilution method. The antibiotics were purchased from Wenzhou Kont Biotechnology Co. Ltd. The MIC of fosfomicin (FOS) was detected by the agar dilution method using medium supplemented with glucose-6-phosphate. The MIC of cefiderocol was

determined using iron-free medium. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were employed as the quality-control isolates. The drug sensitivity results of cefiderocol, MEM, IPM, and ATM were interpreted according to the CLSI M100-34 criteria.<sup>19</sup> The results for TGC were evaluated according to the breakpoint set by the US Food and Drug Administration,<sup>20</sup> and the latest breakpoints from the European Committee on Antimicrobial Susceptibility Testing were applied for AZA and POL.<sup>21</sup> Currently, there are no established sensitivity breakpoints for FOS.

## Phenotype Detection of Carbapenemases

Carbapenemase phenotypes were detected using the enzyme inhibitor enhancement test as previously described.<sup>22</sup> Boric acid (APB) and ethylene diamine tetraacetic acid (EDTA) were purchased from Wenzhou Kont Biotechnology Co. Ltd. The isolates were cultured in the following conditions: A (IPM), B [IPM + APB (400 µg)], C [IPM + EDTA (292 µg)], and D [IPM + APB (400 µg) + EDTA (292 µg)], and the diameters of their respective inhibition zones were measured. The carbapenemase type produced by an isolate was determined according to the inhibition zone diameter as follows: class A carbapenemase - difference between the inhibition zone diameters of disk B and disk A  $\geq 5$  mm; class B metalloenzymes - difference between the inhibition zone diameters of disk C and disk A  $\geq 5$  mm; both class A carbapenemase and class B metalloenzyme - difference between the inhibition zone diameters of disk D and disk A  $\geq 5$  mm; no carbapenemase production - difference between the inhibition zone diameters of disks B, C, and D and that of disk A  $< 5$  mm.

## Next-Generation Sequencing (NGS)

Genomic DNA was extracted from the CRKP<sup>CZA-R</sup> isolates using the Ezup Column Bacterial Genomic DNA Extraction Kit (Shanghai Sangong Bioengineering Co. Ltd.) according to the manufacturer's instructions, and sequenced by Wuhan Baiyi Huineng using the double-ended 150-bp read length dnbseq platform. The raw data were filtered using fastp and subjected to quality control using FastQC. The sequencing data were assembled and corrected using Unicycler (parameter: -1r1-2r2-keep3-modenomal) to obtain the final bacterial genome sequences. The single-nucleotide polymorphisms (SNPs) of the core genomes were identified using Snippy software (version 2, <https://github.com/tseemann/snippy>), and the phylogenetic trees were constructed according to the maximum likelihood method. The MLST types, drug resistance genes, and virulence-related genes of CRKP<sup>CZA-R</sup> isolates were analyzed by BacWGSTdb (BacWGSTdb 2.0, <http://bacdb.cn/BacWGSTdb/>). The capsule locus (KL) types were analyzed by BIGSdb-Pasteur (version 3, <https://bigsdbs.pasteur.fr/>). The phylogenetic trees, MLST types, drug resistance genes, virulence-related genes, and serotypes were visualized using ChiPlot (version 2.6.1, <https://www.chiplot.online/>).

## Results

### Screening and Confirmation of CZA-Resistant CRKP Isolates

In total, 623 non-repetitive CRKP isolates were collected. According to the results of the K-B method, 59 CRKP isolates were identified as CRKP<sup>CZA-R</sup> (inhibition zone  $< 20$  mm) and 539 were confirmed as CZA-sensitive CRKP (inhibition zone  $> 22$  mm). Notably, there were 25 isolates with inhibition zone diameters of 20–22 mm, and the MICs of CZA were further determined by the microdilution broth method. Accordingly, 8 of these 25 unconfirmed isolates (32.0%) were identified as CRKP<sup>CZA-R</sup>, and 17 isolates (68.0%) were identified as CZA-sensitive CRKP. Overall, 67 CRKP<sup>CZA-R</sup> isolates (67/623, 10.8%) were detected.

### Sensitivity of CZA-Resistant CRKP to Other Antimicrobial Agents

We also tested the sensitivity of CRKP<sup>CZA-R</sup> isolates to other antimicrobial agents. The CRKP<sup>CZA-R</sup> isolates were resistant to all carbapenems, and resistance rates to POL, TGC, AZA, cefiderocol were 97.0% (65/67), 83.6% (56/67), 100.0% (67/67), and 94.0% (63/67), respectively. Furthermore, the MIC<sub>50</sub> and MIC<sub>90</sub> for AZA were 0.125/4 µg/mL and 0.5/4 µg/mL, respectively, and that for FOS were 128 µg/mL and  $>512$  µg/mL, respectively. The MIC<sub>50</sub> and MIC<sub>90</sub> for cefiderocol were 2 µg/mL and 4 µg/mL, respectively, although two isolates (C142 and C387) exhibited low-level resistance with MICs of 16 µg/mL.

## Identification of Carbapenemases and Mechanisms of Drug Resistance in CRKP<sup>CZA-R</sup> Isolates

The carbapenemase phenotypes of the 67 CRKP<sup>CZA-R</sup> isolates were tested, and the results indicated that all but one isolate (R2) produced at least one carbapenemase. In addition, 51 isolates were positive only for metalloenzymes, none were positive only for serine enzymes, and 15 isolates were positive for metalloenzymes and serine enzymes.

The predominant carbapenemase-encoding gene in these CRKP<sup>CZA-R</sup> isolates was *bla*<sub>NDM-1</sub> (44.8%, 30/67), followed by *bla*<sub>IMP-4</sub> (9.0%, 6/67) and *bla*<sub>NDM-5</sub> (7.5%, 5/67), and one isolate carried *bla*<sub>NDM-4</sub>. In addition, 37.3% (25/67) of the CRKP<sup>CZA-R</sup> isolates simultaneously harbored more than two types of carbapenemase-encoding genes, of which 21 isolates (31.3%) co-harbored *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>, two isolates simultaneously carried *bla*<sub>KPC-2</sub> and *bla*<sub>IMP-4</sub>, one isolate (R2) carried *bla*<sub>KPC-2</sub> and *bla*<sub>IMP-26</sub>, and one isolate (R57) carried *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>IMP-4</sub>. The outer membrane protein-coding genes, including *ompk35*, *ompk36*, and *ompk37*, were also analyzed. No deletions were detected in any of these genes in 41 isolates (61.2%), while *ompk36* and *ompk35* were deleted in 25 isolates and one isolate, respectively. Furthermore, 21 CRKP<sup>CZA-R</sup> isolates with *ompk36* deletion carried both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>, and 67 CRKP<sup>CZA-R</sup> isolates also carried other drug-resistance genes (Figure 1).

## The Mechanism of Drug Resistance in CRKP Isolates with Inhibition Zone Diameters of 20-22 mm

Twenty-five CRKP isolates with inhibition zone diameters of 20–22 mm to CZA were rechecked by the MIC method. Seven of these isolates, which exhibited an inhibition zone of 20 mm in the K-B test, were confirmed to be CZA-resistant and positive for carbapenemases, and also carried metalloenzyme-encoding genes. In addition, seven isolates had an inhibition zone of 21 mm, of which only one isolate exhibited resistance to CZA through metalloenzyme production and carried the *bla*<sub>IMP-4</sub> gene. The other isolates were sensitive to CZA and produced serinase. Finally, 11 isolates with an inhibition zone of 22 mm were confirmed to be CZA-sensitive after rechecking, and were positive for serinase (Table 1), suggesting that the diameter of the inhibition zone of CZA is between 20–22 mm using the disk diffusion method. Therefore, in case there is no condition to detect the MIC of CZA in clinical laboratories, the detection of carbapenemase types may provide an important reference for drug selection, that is, if serinase carbapenemase is produced, CZA can be used for treatment, but if metalloenzyme is produced, it cannot be used alone.

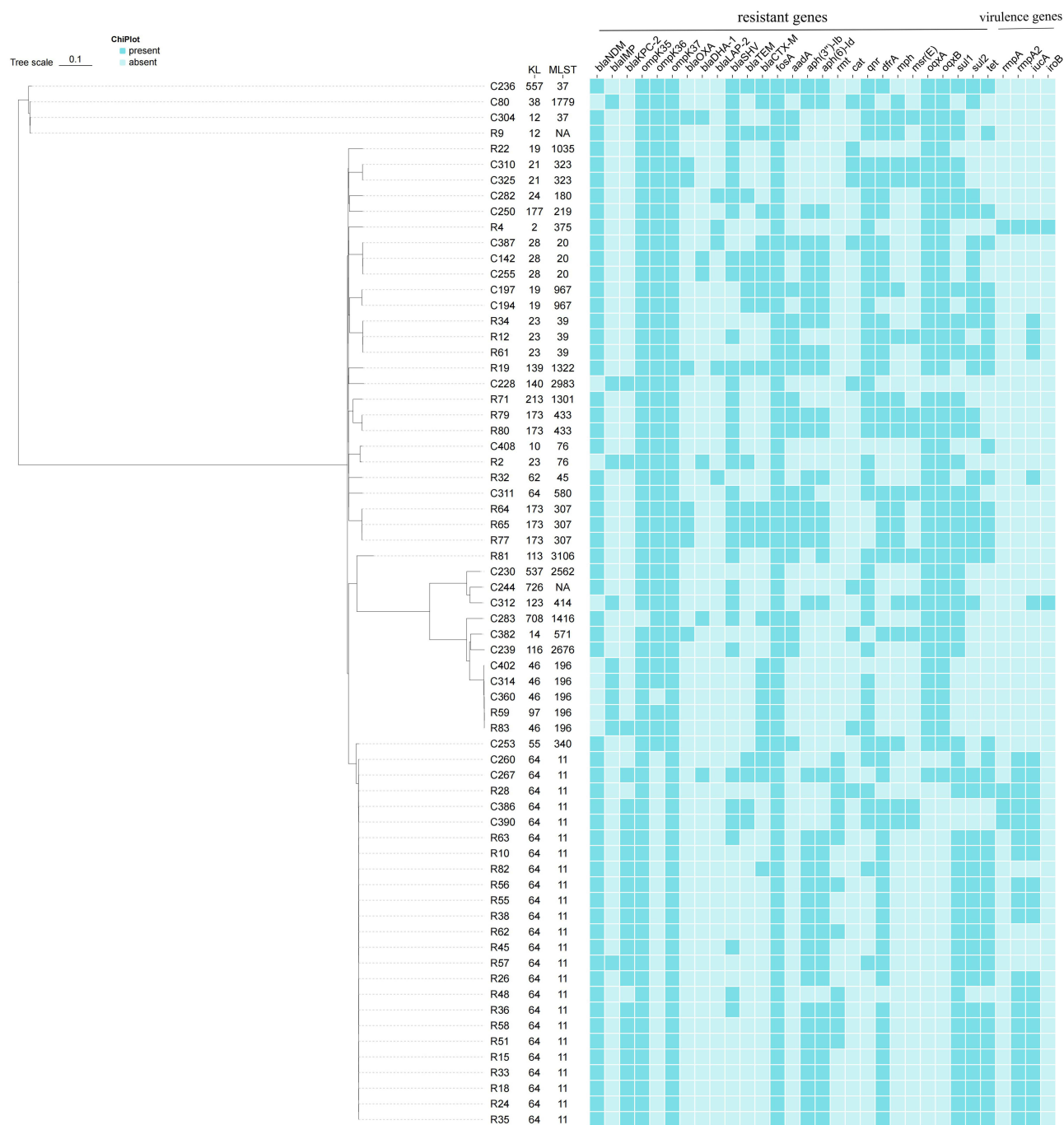
## Homology Analysis

The results of MLST identified 27 STs among the 67 CRKP<sup>CZA-R</sup> isolates, whereas two isolates were not typed. ST11 was the most common type (35.8%, 24/67), followed by ST196 (7.5%, 5/67), ST20 (4.5%, 3/67), ST39 (4.5%, 3/67), and ST307 (4.5%, 3/67, Figure 1). In addition, 21 KL64-ST11 isolates belonged to the same clone cluster with *ompk36* deletion, and co-harbored *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>. Seventeen of these isolates co-harbored two or more hypervirulent marker genes, suggesting clonal transmission of hypervirulent KL64-ST11 CRKP<sup>CZA-R</sup> isolates within our hospital (Figure 1).

## The Risk of Infection with KL64-ST11 CRKP<sup>CZA-R</sup> Isolates Harboring *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>

Patients infected with CRKP<sup>CZA-R</sup> isolates co-harboring *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>, and with *ompk36* deletion, were generally older than those infected with other drug-resistant isolates. Notably, patients harboring these isolates often experienced more severe illness, resulting in higher ICU admission rates and a greater proportion of patients being discharged without recovery (Table 2).

Interestingly, CRKP<sup>CZA-R</sup> isolates harboring both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>, and other resistance genes, exhibited similar MIC<sub>50</sub> and MIC<sub>90</sub> for IPM, MEM, TGC, POL, and AZA. However, the MIC<sub>50</sub> for FOS ranged from 64 to 256 µg/mL, whereas no notable difference was observed in the MIC<sub>90</sub>. The MIC<sub>50</sub> and MIC<sub>90</sub> of cefiderocol for CRKP<sup>CZA-R</sup> carrying both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> were lower compared to that for other isolates, and no intermediate or resistant isolates were identified, suggesting a potentially higher sensitivity to cefiderocol (Table 3).



**Figure 1** Phylogenetic tree, STs, resistance genes, KL types, and virulence-related genes in the 67 CRKP<sup>CZA-R</sup> isolates.

**Abbreviations:** KL, capsule locus; MLST, multilocus sequence typing; NA, not determined.

We detected 27 serotypes, of which KL64 accounted for 37.3% of the isolates (25/67), followed by KL173 (7.5%, 5/67), KL23 (6.0%, 4/67), and KL46 (6.0%, 4/67). The marker genes of hypervirulent isolates, including *rmpA*, *rmpA2*, *iroB*, and *iucA*, were also analyzed. As shown in Figure 1, 32.8% (22/67) of the CRKP<sup>CZA-R</sup> isolates carried two or more hypervirulent marker genes, and four isolates carried *iucA*. None of these marker genes were detected in the remaining isolates. It is worth noting that 17/21 CRKP<sup>CZA-R</sup> isolates carrying *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> also harbored two or more hypervirulent marker genes and exhibited high virulence characteristics (Figure 1).

**Table 1** The Drug Sensitivity, Carbapenemase Phenotypes, and Genotypes of 25 CRKP Isolates Should Be Rechecked by the MIC Method

Isolate Number	Inhibition Zone (mm)	MIC ( $\mu\text{g/mL}$ )	Types of Carbapenemases		Carbapenemase Genotype
			Serinasases	Metalloenzymes Carbapenemases	
C310	20	128/4	–	+	<i>bla<sub>NDM-1</sub></i>
R12	20	32/4	–	+	<i>bla<sub>NDM-1</sub></i>
R64	20	128/4	–	+	<i>bla<sub>NDM-1</sub></i>
R65	20	64/4	–	+	<i>bla<sub>NDM-1</sub></i>
R71	20	64/4	–	+	<i>bla<sub>NDM-1</sub></i>
R77	20	>128/4	–	+	<i>bla<sub>NDM-1</sub></i>
R19	20	64/4	–	+	<i>bla<sub>NDM-4</sub></i>
R59	21	64/4	–	+	<i>bla<sub>IMP-4</sub></i>
C79	21	4/4	+	–	/
C89	21	4/4	+	–	/
C105	21	8/4	+	–	/
C123	21	8/4	+	–	/
C227	21	4/4	+	–	/
C231	21	2/4	+	–	/
C46	22	4/4	+	–	/
C57	22	4/4	+	–	/
C63	22	2/4	+	–	/
C118	22	4/4	+	–	/
C120	22	2/4	+	–	/
C178	22	2/4	+	–	/
C187	22	2/4	+	–	/
C266	22	1/4	+	–	/
C285	22	2/4	+	–	/
C294	22	2/4	+	–	/
C322	22	1/4	+	–	/

Notes: “+”: positive; “–”: negative; “/”: not detected.

**Table 2** Clinical Characteristics of Patients Infected with CRKP<sup>CZA-R</sup> with *bla<sub>KPC-2</sub>* + *bla<sub>NDM-1</sub>* and Others

Clinical Characteristics	CRKP <sup>CZA-R</sup> (n = 67)		<i>bla<sub>KPC-2</sub></i> + <i>bla<sub>NDM-1</sub></i> (n = 21)		Others (n = 46)	
	N	%	N	%	N	%
Age, years (median)	37.3		46.2		35.4	
Sex						
Male	50	74.6	18	85.7	32	69.6
Female	17	25.4	3	14.3	14	30.4
<b>Underlying diseases</b>						
Hypertension	24	35.8	9	42.9	15	32.6
Pulmonary infection	16	23.9	5	23.8	11	23.9
Diabetes	15	22.4	6	28.6	9	19.6
Respiratory failure	6	9.0	2	9.5	4	8.7
Total hospitalization (days)	29.6		27.7		33.6	
ICU admission after infection	40	59.7	16	76.2	24	52.2

(Continued)

Table 2 (Continued).

Clinical Characteristics	CRKP <sup>CZA-R</sup> (n = 67)		<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub> (n = 21)		Others (n = 46)	
	N	%	N	%	N	%
<b>Use of antibiotics before infection</b>						
Piperacillin-tazobactam	25	37.3	11	52.4	14	30.4
Cefoperazone-sulbactam	21	31.3	5	23.8	16	34.8
Meropenem	19	28.4	5	23.8	14	30.4
Vancomycin	14	20.9	4	19.0	10	21.7
Levofloxacin	11	16.4	0	0.0	11	23.9
<b>Pre-infection invasive procedures</b>						
Central venous catheter	38	56.7	14	66.7	24	52.2
Arterial catheterization	35	52.2	13	61.9	22	47.8
Mechanical ventilation	15	22.4	3	14.3	12	26.1
Urinary catheter	14	20.9	5	23.8	9	19.6
<b>Use of antibiotics after infection</b>						
Meropenem	11	16.4	4	19.0	7	15.2
Piperacillin-tazobactam	10	14.9	4	19.0	6	13.0
Cefoperazone-sulbactam	9	13.4	3	14.3	6	13.0
Imipenem	6	9.0	3	14.3	3	6.5
<b>Outcomes</b>						
Discharged without recovery	19	28.4	7	33.3	12	26.1
Death during hospitalization	4	6.0	0	0.0	4	8.7

Table 3 Drug Sensitivity for CRKP<sup>CZA-R</sup> with *bla*<sub>KPC-2</sub> + *bla*<sub>NDM-1</sub> and Others

	S (%)	I (%)	R (%)	MIC <sub>50</sub> (μg/mL)	MIC <sub>90</sub> (μg/mL)
CZA	0.0	/	100.0	64/4	>128/4
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	0.0	/	100.0	128/4	>128/4
Others	0.0	/	100.0	64/4	>128/4
IPM	0.0	0.0	100.0	>32	>32
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	0.0	0.0	100.0	>32	>32
Others	0.0	0.0	100.0	>32	>32
MEM	0.0	0.0	100.0	32	>32
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	0.0	0.0	100.0	32	>32
Others	0.0	0.0	100.0	32	>32
Cefiderocol	94.0	0.0	6.0	2	4
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	100.0	0.0	0.0	1	2
Others	91.4	4.3	4.3	2	4
AZA	100.0	0.0	0.0	0.125/4	0.5/4
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	100.0	0.0	0.0	0.125/4	0.5/4
Others	100.0	0.0	0.0	0.125/4	0.5/4
TGC	83.6	13.4	3.0	2	4
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	85.7	9.5	4.8	2	4
Others	82.6	15.2	2.2	2	4
POL	97.0	0.0	3.0	1	2
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	95.2	0.0	4.8	1	2
Others	97.8	0.0	2.2	1	2
FOS	/	/	/	128	>512
<i>bla</i> <sub>KPC-2</sub> + <i>bla</i> <sub>NDM-1</sub>	/	/	/	256	>512
Others	/	/	/	64	512

Note: "/" indicates that there is no such sensitivity judgment breaking point of the drug.

Abbreviations: S, susceptible; I, intermediate; R, resistant; CZA, ceftazidime-avibactam; IPM, imipenem; MEM, meropenem; AZA, aztreonam-avibactam; TGC, tigecycline; POL, polymyxin B; FOS, fosfomicin.

## Discussion

The global emergence of CRKP in recent years has posed significant challenges to the treatment of bacterial infections.<sup>1–3,23</sup> CZA, a novel  $\beta$ -lactamase inhibitor, has been recommended by the IDSA for the treatment of CRKP infections.<sup>6</sup> However, its widespread use in clinics has led to the emergence of CZA-resistant isolates worldwide. In this study, we screened CRKP<sup>CZA-R</sup> isolates from the clinical isolates of CRKP collected at our hospital from January 1, 2018 to October 30, 2021. In addition, the clinical data of patients infected with CRKP<sup>CZA-R</sup>, and the drug resistance phenotypes and genotypes of these isolates were analyzed to clarify the clinical and molecular epidemiological characteristics of CRKP<sup>CZA-R</sup> infections.

CLSI recommends the K-B method and MIC test for detecting the sensitivity of bacterial isolates to CZA.<sup>19</sup> Given its simplicity and affordability, the K-B method is more appropriate for screening CZA-resistant isolates, particularly in developing countries. Conversely, the MIC method is primarily used to confirm CZA resistance. In this study, 25 isolates with inhibition zone diameters of 20–22 mm in the K-B test were re-evaluated using the microdilution broth method; the isolates with an inhibition zone diameter of 20 mm were confirmed to be CZA-resistant, whereas those with zones measuring 22 mm were sensitive to CZA.<sup>23</sup> However, one isolate with an inhibition zone of 21 mm was resistant to CZA, and it carried the *bla*<sub>IMP-4</sub> gene and tested positive for carbapenemase. The sensitivity of this isolate could be attributed to PcW, an upstream promoter of *bla*<sub>IMP-4</sub>, which likely downregulated the carbapenemase gene.<sup>24</sup> Therefore, in regions where CZA is not widely utilized, further verification using the MIC method is not required when the diameter of the inhibition zone of CZA is 20 or 22 mm. However, verification may be necessary when the diameter is 21 mm.

Since the treatment plans for carbapenem-resistant isolates depend on the types of carbapenemases, IDSA recommends clinical testing for carbapenem-resistant Enterobacterales (CRE).<sup>6</sup> Enzyme inhibitor enhancement is a simple and cost-effective method for detecting carbapenemase phenotypes, and has significant clinical applicability.<sup>22</sup> In our study, this method could detect carbapenemase activity with high sensitivity (82.1%), particularly for isolates harboring a single carbapenemase-encoding gene (100.0%). Nevertheless, its detection capability was limited when dealing with isolates carrying two or more carbapenemase-encoding genes. Furthermore, 10 *bla*<sub>KPC-2</sub> + *bla*<sub>NDM-1</sub> isolates and one *bla*<sub>KPC-2</sub> + *bla*<sub>NDM-5</sub> isolate were positive for metalloenzymes in the carbapenemase phenotype test. It is worth noting that the R2 isolate co-harbored *bla*<sub>KPC-2</sub> and *bla*<sub>IMP-26</sub>, but tested negative for all carbapenemases. Therefore, in clinical practice, it is not feasible to rely solely on enzyme types to determine CZA sensitivity, particularly when the test results are negative. A comprehensive assessment should incorporate drug sensitivity results along with carbapenemase and genotype information.

MBL production is the primary mechanism of CZA resistance. In recent years, new mechanisms of drug resistance have been discovered, such as the presence of new subtypes of KPCs, and the absence of outer membrane proteins.<sup>11,14,16,17</sup> In this study, all CRKP<sup>CZA-R</sup> isolates carried MBL-encoding genes, most commonly *bla*<sub>NDM-1</sub> (44.8%, 30/67), followed by *bla*<sub>IMP-4</sub> (9.0%, 6/67), *bla*<sub>NDM-5</sub> (7.5%, 5/67), and *bla*<sub>NDM-4</sub> (1.5%, 1/67). This suggests that MBL production is the primary mechanism underlying CZA resistance in the CRKP isolates of our hospital. The new subtypes of KPC were not detected in our hospital, which may be attributed to the fact that CZA was not widely used in our hospital at that time. It was worth noting that 35.8% (24/67) of the CRKP<sup>CZA-R</sup> isolates simultaneously carried more than two carbapenemase-encoding genes, of which 21 isolates co-harbored *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>, two isolates carried *bla*<sub>KPC-2</sub> and *bla*<sub>IMP-4</sub>, one isolate (R2) carried *bla*<sub>KPC-2</sub> and *bla*<sub>IMP-26</sub>, and one isolate (R57) carried *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>IMP-4</sub>. Recent reports indicate a rise in multi-carbapenemase-producing isolates, which would pose a serious threat to public health.<sup>25,26</sup>

At present, there is a lack of effective and reliable antibacterial drugs for nosocomial CRKP<sup>CZA-R</sup> infections. The CRKP<sup>CZA-R</sup> isolates from our hospital were highly sensitive to POL and TGC. Although studies indicate that this drug combination might be effective against CRKP<sup>CZA-R</sup>, POL is associated with heterogeneous resistance and nephrotoxicity, and TGC has poor efficacy in treating severe infections, thereby precluding their use for CRKP<sup>CZA-R</sup> infections.<sup>6,27</sup> In addition, some reports suggest that the combination of FOS and other antibiotics can be used to treat urinary tract infections.<sup>28</sup> Currently, metalloenzyme-producing CRKP is clinically managed using the combination of ATM (which has good stability against MBLs<sup>29</sup>) and CZA. Nevertheless, this therapeutic regimen is not effective against all MBL-producing isolates, which might be associated with mutations arising at specific sites within

PBP3, the target site of ATM.<sup>30</sup> Recently, AZA, a novel composite antibacterial agent, has gained approval for market release in Europe. The MIC of AZA in this study was exceptionally low, thus demonstrating its immense potential in the treatment of CRKP<sup>CZA-R</sup> infections.<sup>31</sup> In addition, cefiderocol monotherapy has been recommended by IDSA for the treatment of metalloenzyme-producing CRKP.<sup>6</sup> Novel antibiotics such as cefepime-taniborbactam may also be sensitive to metalloenzyme-producing CRKP.<sup>32</sup> In this study, the detection rate of *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub> was higher. For these strains, the combination of ATM with innovative  $\beta$ -lactam/ $\beta$ -lactamase inhibitors could prove to be an effective treatment option.<sup>33</sup>

ST11 was the predominant ST, and accounted for 35.8% (24/67) of the CRKP<sup>CZA-R</sup> isolates. Notably, 17 ST11 CRKP<sup>CZA-R</sup> isolates concurrently harbored both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> along with *ompk36* deletion, in addition to two or more hypervirulent marker genes. These findings are indicative of the clonal transmission of KL64 ST11 hypervirulent CRKP<sup>CZA-R</sup> isolates within our hospital. Furthermore, patients infected with these isolates were older than those infected with other drug-resistant isolates, and exhibited more serious illness. The ICU occupancy rate of these patients was also higher, as was the proportion of patients who were discharged without recovery. In addition, the MIC<sub>50</sub> of FOS was significantly higher for the KL64 ST11 CRKP isolates co-harboring *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> than for other drug-resistant isolates, suggesting that FOS should be used cautiously in the treatment of urinary tract infections caused by these isolates. Remarkably, cefiderocol exhibited lower MIC<sub>50</sub> and MIC<sub>90</sub> for isolates co-harboring *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> compared to other isolates, and no intermediate or resistant isolates were identified, suggesting a potentially higher sensitivity to cefiderocol. Previous studies revealed that the prevalent serotype associated with ST11 CRKP has shifted from KL47 to primarily KL64, which might be related to factors such as the superior antioxidant capabilities of the KL64 isolate, or recombination.<sup>1,34-36</sup> Furthermore, KL64-ST11 CRKP exhibits remarkable genetic plasticity. Although IncFII/IncR plasmids carrying the *bla*<sub>KPC-2</sub> gene cannot be transferred independently, they can form hybrid plasmids with IncC plasmids harboring *bla*<sub>NDM</sub>, thereby facilitating transfer.<sup>26</sup> In this study, the isolates harboring these genes also featured *ompk36* deletion. It remains to be confirmed whether *ompk36* deletion facilitates the acquisition of drug resistance genes through horizontal transfer. Altogether, our findings suggest that the clonal transmission of KL64-ST11 CRKP in hospitals should be monitored closely.

## Conclusion

CRKP<sup>CZA-R</sup> isolates had a high detection rate in our hospital, and the main drug resistance mechanism was MBLs production. Nevertheless, these isolates exhibit very high sensitivity to POL, TGC, AZA, and cefdzime. The enzyme inhibitor enhancement method can detect carbapenemases with high sensitivity and specificity and provide an important reference for drug selection, but it might have limited detection ability for isolates that simultaneously produce more than two carbapenemases. Patients infected with CRKP<sup>CZA-R</sup> co-harboring both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> exhibited more serious disease; these isolates exhibit lower sensitivity to FOS, high sensitivity to cefiderocol, and clonal transmission, all of which warrant concern.

## Abbreviations

CZA, Ceftazidime-avibactam; CRKP, *K. pneumoniae*; CRKP<sup>CZA-R</sup>, CZA-resistant CRKP; WHO, World Health Organization; AVI, avibactam; KPC, *K. pneumoniae* carbapenemase; IDSA, Infectious Diseases Society of America; MBL, metallo- $\beta$ -lactamase; ST, sequence type; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MEM, meropenem; IPM, imipenem; ATM, aztreonam; POL, polymyxin B; TGC, tigecycline; FOS, fosfomycin; AZA, aztreonam-avibactam; APB, boric acid; EDTA, Ethylene Diamine Tetraacetic Acid; NGS, Next-generation sequencing; CRE, Carbapenem resistant Enterobacterales.

## Data Sharing Statement

The datasets generated and analyzed during the current study are available in the NCBI database with ID PRJNA1236832. <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1236832>.

## Ethics Approval and Informed Consent

The study was approved by the Clinical Research Medical Ethics Subcommittee of the Medical Ethics Committee of Xiangya Hospital, Central South University, with ID 202209541. All methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all participants.

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## Disclosure

The authors report no conflicts of interest in this work.

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