

# Microbiological Characteristics of Carbapenem-Resistant *Enterobacteriaceae* Clinical Isolates Collected from County Hospitals

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**Objective:** To investigate the molecular characteristics of carbapenem-resistant *Enterobacteriaceae* (CRE) from county hospitals in China.

**Materials and Methods:** Forty-three sequential non-duplicate CRE strains (including 33 *Klebsiella pneumoniae* isolates, 4 *Enterobacter cloacae* isolates, 3 *Escherichia coli* isolates, 1 *Serratia marcescens*, 1 *Morganella morganii* and 1 *Citrobacter freundii*) were collected from 4 county hospitals and 2 municipal hospitals. Antimicrobial susceptibility testing was conducted by broth microdilution method, using 3-aminophenylboronic acid and EDTA and the modified carbapenem inactivation method (mCIM) to screen phenotype of carbapenemase.  $\beta$ -Lactamases were characterized by polymerase chain reaction (PCR) and DNA sequencing. The transferability of *bla*<sub>NDM-5</sub> was investigated by transformation experiment. Clonal relatedness was evaluated by pulsed-field gel electrophoresis and multilocus sequence typing.

**Results:** The results of antimicrobial susceptibility testing indicated that 43 CRE strains were resistant to most of the antimicrobial agents, except tigecycline and colistin. Overall, 93%, 93%, and 97.7% of these strains were resistant to imipenem, meropenem, and ertapenem, respectively. PCR and DNA sequencing indicated that 67.4% (29/43) were *bla*<sub>KPC-2</sub> positive isolates, in which 3.4% (1/29) was coproduced with *bla*<sub>NDM-1</sub>. In addition, 7.0% (3/43), 4.7% (2/43), 4.7% (2/43), 2.3% (1/43), 2.3% (1/43) were *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-16</sub>, *bla*<sub>NDM-4</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>IMP-4</sub> positive isolates, respectively. The 29 *bla*<sub>KPC-2</sub>-positive isolates belonged to 12 different PFGE type and designated as ST11 (n=20) and ST15, ST39, ST116, ST667, ST2245, ST2338. The plasmid bearing *bla*<sub>NDM-5</sub> could be transferred into recipient *E. coli* J53 through transformation.

**Conclusion:** Our study indicated the dissemination of CRE between the tertiary hospitals and secondary hospitals.

**Keywords:** *Enterobacteriaceae*, *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub>

## Introduction

Carbapenems are often used as a last resort to treat Gram-negative bacteria infections. The emergence and rapid spread of carbapenemases in *Enterobacteriaceae* clinical isolates which were the most frequent pathogens of causing nosocomial infections is becoming a great public health problem worldwide.<sup>1,2</sup> According to the CHINET surveillance network reported ([www.chinets.com](http://www.chinets.com)), the rates of *Klebsiella pneumoniae* resistance to imipenem and meropenem increased markedly from 2005 to 2014, from 1.3% to 14.6% and from 0% to 15.2%, respectively.<sup>3</sup> Currently, three

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main classes of carbapenemases have been identified including class A, B, and D type carbapenemases (these groups do not solely contain carbapenemases). *Klebsiella pneumoniae* carbapenemases (KPC) was first reported in the United States in the late 1990s and since then worldwide, with a marked endemicity in the United States, Greece, and Italy.<sup>4,5</sup>

Because genes encoding carbapenemase are often located in plasmids, and these plasmids often carry mobile genetic elements and a variety of resistance genes, causing resistance to spread between different bacteria, different cities and different countries. Currently, the study about carbapenemases mainly focused on the *Enterobacteriaceae* clinical isolates collected from large cities and hospitals, rarely involving county hospitals. Our study aimed to investigate the prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) clinical isolates in county hospital and to provide basis for prevention and control of nosocomial infections.

## Materials and Methods

### Bacterial Isolates

A sequential non-duplicated collection of 43 CRE (33 *Klebsiella pneumoniae* isolates, 4 *Enterobacter cloacae* isolates, 3 *Escherichia coli* isolates, 1 *Serratia marcescens*, 1 *Morganella morganii* and 1 *Citrobacter freundii*) were routine obtained in clinical microbiology laboratory from January to June in 2017. From 4 county hospitals (usually called secondary hospital, the numbers range of beds is 200–480) and 2 municipal hospitals (usually called municipal hospital, the numbers of beds is 1300 and 1600, respectively) in Zhejiang province in China. Of 43 isolates, 58.1% (25/43) were isolated from sputum, followed by urine (20.9%, 9/43), blood (11.6%, 5/43), secretion sample (7.0%, 3/43) and abdominal fluid (2.3%, 1/43). All clinical isolates were resistant to at least one of carbapenem (ertapenem, imipenem or meropenem) and were identified using the VITEK 2 Compact system. *E. coli* ATCC 25922, *Salmonella* ser. Braenderup H9812, and *E. coli* J53 were used as the quality control for antimicrobial susceptibility testing, reference marker for PFGE, and recipient strain for transformation, respectively.

### Antimicrobial Susceptibility Testing and $\beta$ -Lactamase Characterization

Antimicrobial susceptibility testing was performed using broth microdilution method and results were interpreted following the criteria of the Clinical and Laboratory

Standards Institute.<sup>6</sup> The breakpoints proposed by European Committee on Antimicrobial Susceptibilities Testing (EUCAST) were used for colistin and tigecycline.<sup>7</sup> The carbapenem inactivation method (CIM) were used for phenotypic detection of carbapenemase.<sup>8</sup> The presence of genes encoding  $\beta$ -lactamase, including CTX-M-type extended-spectrum $\beta$ -lactamases (ESBLs), plasmid-born AmpC  $\beta$ -lactamases, and carbapenemases, were investigated by PCR using primers previously described.<sup>9–11</sup> Polymerase chain reaction (PCR) amplicons were sequenced and the DNA sequences obtained were compared with those available in the NCBI GenBank database using BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Transfer of Carbapenemase Resistance

Transformation experiment was carried out with *E. coli* J53 as the recipient to determine the transferability of the carbapenemase gene, as described previously.<sup>12</sup>

### Bacterial Genotyping

Whole-cell DNA of clinical strains embedded in agarose gel plugs, was separated by PFGE for 33 *K. pneumoniae* isolates and 3 *E. coli* isolates. Clonal relationships were analyzed using PFGE of *Xba*I-digested genomic DNA as previously described,<sup>13</sup> and the results were analyzed according to the criteria proposed by Tenover et al.<sup>14</sup> MLST for these isolates was performed as described previously.<sup>15</sup>

## Results

### Antimicrobial Susceptibility Testing

The results of antimicrobial susceptibility testing indicated the resistant rate of 43 CRE isolates to imipenem, meropenem, and ertapenem was 93%, 93%, and 97.7% with the MIC<sub>50</sub>/MIC<sub>90</sub> of >16/>16 mg/L, >16/>16mg/L and >32/>32 mg/L, respectively. 46.5%, 90.3% and 93.0% were susceptible to amikacin, tigecycline and colistin, respectively (Table 1). For three colistin-resistant strains, one was *K. pneumoniae*, and the other two strains were *Serratia marcescens* and *Morganella morganii* which was intrinsically resistant to colistin.

### $\beta$ -Lactamase Characterization

The result of CIM indicated that 97.7% (42/43) of these CRE isolates were carbapenemase-producing. PCR and DNA sequencing indicated that 67.4% (29/43) were

**Table 1** Resistance and Susceptibility of All the Strains to Antimicrobial Agents

Antimicrobial Agent	%R	%S	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC Range
Piperacillin	97.7	2.3	>256	>256	≤2→256
Cefoperazone-sulbactam	93	4.7	>128	>128	≤1→128
Piperacillin-tazobactam	95.3	2.3	>256	>256	≤2→256
Cefazolin	100	0	>32	>32	>32→32
Cefuroxime	100	0	>64	>64	32→64
Ceftazidime	93	4.7	>32	>32	≤0.25→32
Cefepime	97.7	2.3	>32	>32	≤0.25→32
Cefotaxime	95.3	2.3	>32	>32	≤0.25→32
Cefmetazole	79.1	18.6	>64	>64	4→64
Moxalactam	76.7	9.3	>64	>64	≤0.5→64
Aztreonam	93	7	>128	>128	≤1→128
Ertapenem	97.7	2.3	>32	>32	≤0.25→32
Imipenem	93	0	>16	>16	2→16
Meropenem	93	7	>16	>16	≤0.125→16
Amikacin	51.2	46.5	>128	>128	2→128
Gentamicin	67.4	27.9	>128	>128	≤1→128
Ciprofloxacin	81.4	18.6	>8	>8	≤0.06→8
Levofloxacin	67.4	23.3	16	>16	≤0.125→16
Trimethoprim-sulfamethoxazole	53.5	46.5	>32	>32	≤0.25→32
Colistin	7.0	93	0.25	0.5	≤0.125→16
Nitrofurantoin	67.4	18.6	128	256	8→128
Tigecycline	9.7	90.3	0.5	1	0.25→2

**Abbreviations:** R, resistance; S, susceptible.

*bla*<sub>KPC-2</sub> positive isolates, in which 3.4% (1/29) was coproduced with *bla*<sub>NDM-1</sub>. In addition, 7.0% (3/43), 4.7% (2/43), 4.7% (2/43), 2.3% (1/43), 2.3% (1/43) were *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-16</sub>, *bla*<sub>NDM-4</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>IMP-4</sub> positive isolates, respectively (Table 2). PCR was used to detect genes encoding OXA, and VIM type carbapenemases but did not result in amplicons with any of the clinical isolates. No carbapenemase gene was detected for four isolates in this study and only ESBL or plasmid-mediated AmpC were positive including *bla*<sub>CTX-M-15</sub> (*K. pneumoniae*), *bla*<sub>DHA-1</sub> (*M. morgannii*), *bla*<sub>CMY-2</sub> (*E. coli*) and *bla*<sub>SHV-12</sub> (*E. coli*). Two isolates were negative for all of  $\beta$ -lactamase gene detected in this study.

### Transfer of Carbapenemase Resistance

One *E. coli* bearing *bla*<sub>NDM-5</sub> coupled with *bla*<sub>CTX-M-55</sub> and *bla*<sub>SHV-12</sub> were random selected for transformation experiment with *E. coli* J53 as the recipient. The plasmid bearing *bla*<sub>NDM-5</sub> was successfully transferred from donor

to recipient *E. coli* J53, and the transformation exhibited high resistant to carbapenems, consistent with the detection of *bla*<sub>NDM-5</sub>. The MICs of imipenem, meropenem, and ertapenem for the transformant were >16, >16, 32 mg/L. The MICs of meropenem increased more than 1000 times compared with the recipient.

### Bacterial Genotyping

PFGE shows 12 different types for 33 *K. pneumoniae* isolates, and ST11 (60.6%, 20/33) was the predominant clone. The other ST type were ST2, ST15, ST39, ST58, ST116, ST506, ST667, ST705, ST1110, ST2245, ST2338, ST2536 (Figure 1A). PFGE shows 3 different types for 3 *E. coli* isolates (Figure 1B). For *K. pneumoniae* isolates, most of isolates were dissemination in the same hospital. However, PFGE type G1 (isolated from hospital secondary 3 and tertiary 4), H (isolated from hospital secondary 2 and tertiary 1), K (isolated from hospital secondary 3 and tertiary 1) and O (isolated from hospital secondary 5 and tertiary 4) isolates were dissemination among different hospitals (Figure 1A).

### Discussion

Although carbapenems are the most widely antimicrobial spectrum of antibacterial drugs for *Enterobacteriaceae* clinical isolates, infections caused by CRE associated with significant morbidity and mortality are increasing year by year.<sup>16</sup> In this study, 43 CRE isolates were highly resistant to most common antimicrobial agents except tigecycline, colistin, trimethoprim-sulfamethoxazole and amikacin with the sensitive rate for 90.3%, 93%, 46.5% and 46.5%, respectively. Although more than 90% of isolates were susceptible to tigecycline and colistin, tigecycline is not recommended for bloodstream infection because of the low concentration, and currently colistin is also not available for patients in China. The limitation of available therapeutic regimens for the infection caused by CRE is the great challenge. For the purpose to limit the spread of CRE, early monitoring of CRE infection or colonization on admission may play a more important role for timely control of the spread of CRKP.<sup>17</sup> As Gorrie reported, the carriage frequencies of *K. pneumoniae* were about 6% among ICU patients admitted directly from the community, and 19% among those with recent healthcare contact. Gut colonization on admission was significantly associated with subsequent infection, 49% of *K. pneumoniae* infections were caused by the patients' own unique strain, and 48% of screened patients with infections were positive for prior colonization.<sup>18</sup>

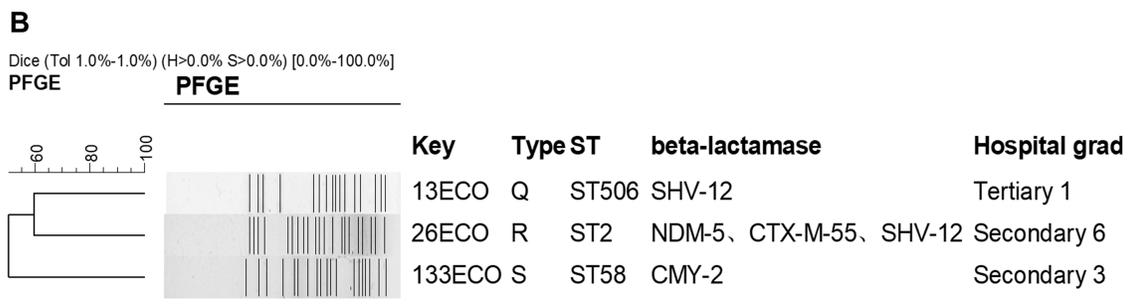
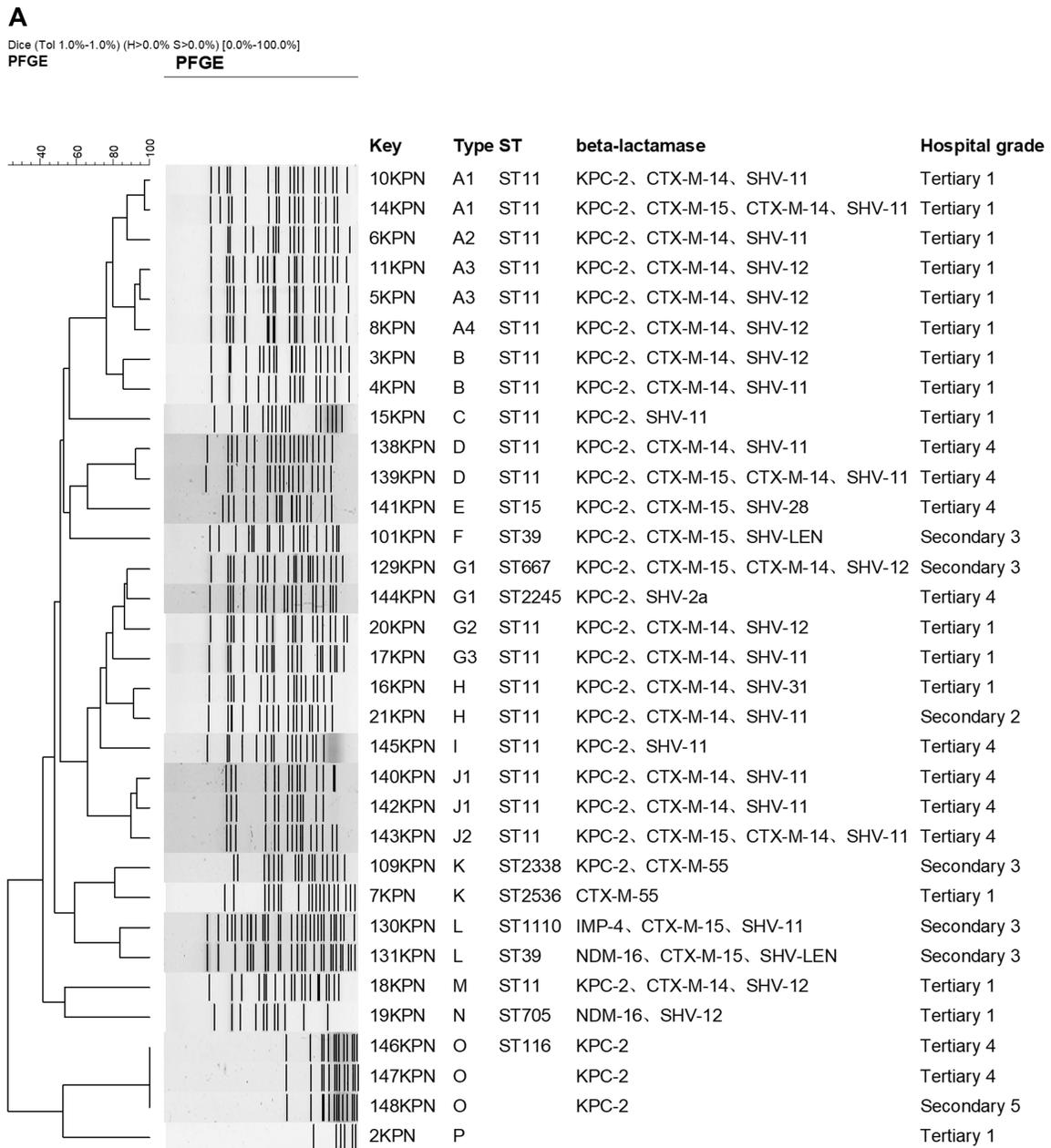
**Table 2** Distribution of Gene Type of 43 Strains of CRE

Gene Type	Strains (Number)	MIC Range (mg/L)				PFGE	MLST
		Imipenem	Meropenem	Colistin	Tigecycline		
KPC-2+CTX-M-14+SHV-11	<i>K. pneumoniae</i> (8)	8->16	8->16	<=0.125-1	0.25-1	A1,A2,B,D,G3,H, J1(2)	ST11(8)
KPC-2+CTX-M-14+SHV-12	<i>K. pneumoniae</i> (6)	>16	>16	0.25-0.5	0.25-2	A3(2),A4,B,G2,M	ST11(6)
KPC-2+CTX-M-15+CTX-M-14+SHV-11	<i>K. pneumoniae</i> (3)	8->16	16->16	0.25-0.5	0.25-1	A1,D,J2	ST11(3)
KPC-2	<i>K. pneumoniae</i> (3)	>16	8->16	<=0.125->16	0.25-2	O(3)	ST116(1)
KPC-2+SHV-11	<i>K. pneumoniae</i> (2)	16->16	>16	0.25	0.5-1	C,I	ST11(2)
NDM-4	<i>E. cloacae</i> (2)	4-4	8-8	0.25	0.25-0.5	-	-
CTX-M-55	<i>K. pneumoniae</i> (1)	32	>16	0.25	0.25	K	ST2536
DHA-1	<i>M. organii</i> (1)	4	<=0.125	>16	1	-	-
CMY-2	<i>E. coli</i> (1)	2	1	0.25	0.5	S	ST58
KPC-2+CTX-M-55	<i>K. pneumoniae</i> (1)	>16	>16	0.25	0.5	K	ST2338
KPC-2+SHV-2a	<i>K. pneumoniae</i> (1)	>16	>16	0.25	2	G1	ST2245
KPC-2+CTX-M-15+SHV-LEN	<i>K. pneumoniae</i> (1)	>16	>16	0.25	0.25	F	ST39
KPC-2+CTX-M-14+SHV-31	<i>K. pneumoniae</i> (1)	>16	>16	0.25	0.5	H	ST11
KPC-2+CTX-M-15+SHV-28	<i>K. pneumoniae</i> (1)	8	8	0.25	0.25	E	ST15
KPC-2+CTX-M-15+CTX-M-14+SHV-12	<i>K. pneumoniae</i> (1)	>16	>16	0.25	0.25	G1	ST667
KPC-2+NDM-1+SHV-12	<i>C. freundii</i> (1)	8	8	0.5	1	-	-
SHV-12	<i>E. coli</i> (1)	4	1	0.5	0.25	Q	ST506
NDM-1	<i>E. cloacae</i> (1)	4	16	0.25	0.25	-	-
NDM-1+SHV-12	<i>E. cloacae</i> (1)	2	4	<0.125	0.25	-	-
NDM-5+CTX-M-55+SHV-12	<i>E. coli</i> (1)	>16	>16	0.5	0.5	R	ST2
NDM-16+SHV-12	<i>K. pneumoniae</i> (1)	8	16	0.25	0.5	N	ST705
NDM-16+CTX-M-15+SHV-LEN	<i>K. pneumoniae</i> (1)	8	16	0.25	0.5	L	ST39
IMP-4+CTX-M-15+SHV-11	<i>K. pneumoniae</i> (1)	2	4	0.25	0.25	L	ST1110
Negative	<i>K. pneumoniae</i> (1), <i>S. marcescens</i> (1)	>16	16->16	0.5->16	0.25-2	-	-

Studies had shown that the resistance mechanism of carbapenem-resistant *Enterobacteriaceae* clinical strains isolated from children patients, adult patients or from the different geographical distribution were the difference.<sup>19</sup> In China, for the CRE strains isolated from children patients, the main type of carbapenemases mediated resistance to carbapenems was metallo- $\beta$ -lactamases including *bla*<sub>NDM-1</sub> and *bla*<sub>IMP</sub>, while *bla*<sub>KPC</sub> was predominant among adult patients. However, the diversity of carbapenemases among CRE isolated from county hospitals and municipal hospitals was not unequivocal. Our study indicated 67.4% (29/43) were *bla*<sub>KPC-2</sub> positive isolates, in which 3.4% (1/29) was coproduced with *bla*<sub>NDM-1</sub>. 20.9% (9/43) were producing metallo- $\beta$ -lactamases including *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-16</sub>, *bla*<sub>NDM-4</sub>, *bla*<sub>NDM-5</sub>, and *bla*<sub>IMP-4</sub>. For 26 *K. pneumoniae* clinical strains collected from municipal hospitals, 3.8% (1/26) was *bla*<sub>NDM-16</sub> positive. However, 28.6% (2/7) was *bla*<sub>NDM-16</sub> positive or *bla*<sub>IMP-4</sub> positive isolates among 7 *K. pneumoniae* clinical

strains collected from county hospitals. The diversification of carbapenemases among CRE from different patients may strengthen the difficulty for infection control to limit the spread of CRE in clinical facilities.<sup>20-22</sup> In this study, we also studied the relation for the CRE strains collected from tertiary hospitals and secondary hospitals. PFGE shows 12 different types for 33 *K. pneumoniae* isolates, and ST11 (60.6%, 20/33) was the predominant clone which was the main ST type for CRE. Except for one strain was isolated from secondary hospital, the other ST11 CRE were all from tertiary hospitals.

As reported,<sup>23-25</sup> ST11 *K. pneumoniae* clone is the dominant clone of KPC-producing *K. pneumoniae* in the world. ST11 clone would be good colonizers to capture plasmids and these isolates with ST11 clone will be easily transmitted between patients. For controlling the dissemination of CRE especially for ST11 KPC-producing *K. pneumoniae* among health-care facilities, rapid and accuracy detection of carbapenemases is critical for



**Figure 1** DNA fingerprints and β-lactamases distribution of *K. pneumoniae* isolates (A) and *E. coli* isolates (B).

preventing and controlling outbreaks of CRE, however, there is no perfect method is suitable for detecting all types of carbapenemases because some carbapenemase producers demonstrate low resistance level to carbapenems.<sup>26,27</sup> Currently, mCIM method was recommended by CLSI to detect the suspected carbapenemases among *Enterobacteriaceae* clinical isolates. Although mCIM has high sensitivity and specificity for confirmation of carbapenemase-producing strains, it is time-consuming for incubating 18–20h to read the final result and the false negative for some carbapenemase including OXA-type carbapenemase. Currently, several molecular technologies for Rapid detection of CRE, particularly carbapenemase-producing CRE have been developed for the automated detection of target genes including class A, class B and class D type carbapenemases directly from the clinical samples with high sensitivity and specificity,<sup>28</sup> however, it only can detect the known gene, nor for the novel carbapenemase gene. In the future, we should investigate the novel carbapenemase gene for 6 strains which were carbapenemase-negative isolates determined by PCR including one was negative by mCIM.

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## Disclosure

The 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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