

# Carbapenem-Resistant *Enterobacter cloacae* Complex in Southwest China: Molecular Characteristics and Risk Factors Caused by NDM Producers

Zhangrui Zeng<sup>1,\*</sup>, Yueshuai Wei<sup>1,\*</sup>, Caihong Ye<sup>1</sup>, Yuan Jiang<sup>1</sup>, Chunlin Feng<sup>1</sup>, Tongtong Guo<sup>1</sup>, Min Song<sup>1</sup>, Yinhan Ding<sup>1</sup>, Ping Zhan<sup>2</sup>, Jinbo Liu<sup>1</sup>

<sup>1</sup>Department of Laboratory Medicine, The Affiliated Hospital of Southwest Medical University, Sichuan Province Engineering Technology Research Center of Molecular Diagnosis of Clinical Diseases, Molecular Diagnosis of Clinical Diseases Key Laboratory of Luzhou, Luzhou, People's Republic of China; <sup>2</sup>Department of Gynaecology, The Affiliated Hospital of Southwest Medical University, Luzhou, People's Republic of China

\*These authors contributed equally to this work

Correspondence: Jinbo Liu, Department of Laboratory Medicine, The Affiliated Hospital of Southwest Medical University, Sichuan Province Engineering Technology Research Center of Molecular Diagnosis of Clinical Diseases, Molecular Diagnosis of Clinical Diseases Key Laboratory of Luzhou, Luzhou, People's Republic of China, Te/Fax +86 0830 3165730, Email liujb7203@swmu.edu.cn

**Purpose:** The isolation rate of carbapenem-resistant *Enterobacter cloacae* complex (CREC) is continuously increasing. The aims of this study were to investigate the molecular characteristics and risk factors associated with CREC infections.

**Methods:** Bacterial species were identified using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany), and the *hsp60* gene was utilized for further typing. Antimicrobial susceptibilities were assessed through the MicroScan WalkAway 96 Plus system (Siemens, Germany) and the microbroth dilution method. Antimicrobial resistance genes were screened through polymerase chain reaction (PCR), while the homologous relationship was assessed using multilocus sequence typing (MLST). Conjugation experiments were performed to verify whether the plasmid could be transferred. Additionally, logistic regression model was employed to analyze risk factors for CREC infections.

**Results:** 32 strains of CREC bacteria were isolated during the study, yet only 20 were retained for preservation. While the isolates demonstrated resistance to the majority of antibiotics, they exhibited high sensitivity to polymyxin B and tigecycline. All isolates carried the *bla*<sub>NDM</sub> resistance gene, including 13 *bla*<sub>NDM-1</sub> isolates and 7 *bla*<sub>NDM-5</sub> isolates. MLST homology analysis revealed the presence of seven known ST types and one new ST type. Conjugation experiments confirmed that 13 isolates were capable of transferring the *bla*<sub>NDM</sub> resistance gene to *Escherichia coli* strain EC600. Single-factor analysis identified multiple primary risk factors for CREC infection, but multivariate analysis did not reveal independent risk factors.

**Conclusion:** This study investigates the molecular characteristics and risk factors associated with CREC infections. The detection rate of CREC strains in our hospital is continuously rising and homology analysis suggested that strains might spread in our hospital, emphasizing the importance of implementing effective preventive measures to control the horizontal transmission of plasmid-mediated antimicrobial resistance genes.

**Keywords:** carbapenem-resistant, *Enterobacter cloacae* complex, antimicrobial resistance, molecular characteristics, risk factors

## Introduction

*Enterobacter cloacae* complex (ECC), a species of the *Enterobacteriaceae* family, is increasingly prevalent in clinical infections. They are widespread in the environment and represent a clinically significant opportunistic pathogen.<sup>1</sup> ECC comprises various species including *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter asburiae*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter nimipressuralis*, *Enterobacter mori*, among others.<sup>2</sup> The *hsp60* gene typing scheme serves as a common method for distinguishing members within ECC, and it is widely adopted in practice.<sup>3</sup> ECC strains are notorious members of the “ESKAPE” pathogens, causing diverse infections in clinical

settings such as pulmonary, urinary system, and bloodstream infections.<sup>4</sup> Recent studies have shown that this emerging multidrug-resistant (MDR) pathogen can lead to severe infections in newborns, resulting in poorer prognosis and higher treatment costs.<sup>5,6</sup>

With the increase of prevalence of clinical strains that producing extended-spectrum  $\beta$ -lactamase (ESBL), carbapenem antibiotics are regarded as the last line of defense against MDR bacterial infections. However, the clinical isolation rate of carbapenem-resistant *Enterobacteriaceae* (CRE) is gradually increasing.<sup>7</sup> According to the report from the China Antimicrobial Resistance Surveillance System (CHINET), the resistance rate of *Enterobacteriaceae* to carbapenems was 2.7% in 2012. However, over the span of ten years, this rate has astonishingly risen to 9.7% in 2022. Mechanisms such as the production of carbapenemases, overexpression of efflux pumps, and mutation of porins contribute to *Enterobacteriaceae* resistance to carbapenem antibiotics.<sup>8</sup> Carbapenemases production play a crucial role as the primary mechanism contributing to bacterial resistance, including enzymes such as KPC, NDM, VIM, IMP, and OXA-48.<sup>9</sup> New Delhi metallo- $\beta$ -lactamase (NDM) is an important member of class B carbapenemase, existing in various subtypes, with NDM-1 being the most common.<sup>10</sup> The production of these enzymes renders most antibiotic treatments ineffective, posing a significant challenge to public health systems.

This retrospective study was conducted in a tertiary hospital in Southwest China with the aims of identifying the clinically isolated species of CREC strains, investigating the molecular characteristics and risk factors associated with CREC infections. The findings from this study will provide valuable insights for the hospital to control and prevent CREC infections.

## Materials and Methods

### Bacterial Collection

CREC was defined as *Enterobacter cloacae* complex strains resistant to imipenem or meropenem. From January 2020 to January 2023, a total of 32 strains of CREC bacteria were isolated, however, only 20 strains were preserved. Then, clinical isolates were identified using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany). Because of the genetic similarity of *Enterobacter cloacae* complex members, amplification of the *hsp60* gene was performed for the further identification of bacterial species.<sup>3</sup> The primers were shown in [Table S1](#).

### Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing on CREC isolates was performed using the MicroScan WalkAway 96 Plus system (Siemens, Germany) and the microbroth dilution method, including Amikacin, Imipenem, Meropenem, Cefazolin, Cefuroxime, Ceftriaxone, Cefotaxime, Ceftazidime, Cefepime, Sulperazon, Ciprofloxacin, Levofloxacin, Tobramycin, Aztreonam, Ampicillin/sulbactam, Piperacillin/tazobactam, Cefoxitin, Tigecycline and Polymyxin B. The interpretation of the results followed the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). For the interpretation of tigecycline susceptibility results, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were employed. *Escherichia coli* ATCC25922 was used as the control strain, obtained from the China National Health Inspection Center.

### Screening of CREC and Detection of Antimicrobial Resistance Genes

To verify whether the strain produces carbapenemases, as previously described, modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM) were utilized for validation.<sup>11</sup> The phenotypic screening was conducted following the guidelines provided by the CLSI. Bacterial DNA templates were obtained through bacterial boiling, and polymerase chain reaction (PCR) was conducted to identify antimicrobial resistance genes (*bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>OXA-48</sub>). Additionally, the presence of ESBL and AmpC resistance genes was also assessed. Positive samples were sent to Shenggong Biotechnology Co., Ltd. (Shanghai, China) for sanger sequencing, and the obtained sequencing results were analyzed through the NCBI BLAST database

(<http://www.ncbi.nlm.nih.gov/BLAST/>). The primers employed to target resistance genes were referenced from the previous protocol.<sup>12</sup> The primers were shown in [Table S2](#).

## Plasmid Conjugation Test

To validate whether the resistance genes are located on plasmids and capable of horizontal transfer, conjugation experiments were carried out. Following the previous experimental protocol with slight modifications, CREC strains carrying the *bla*<sub>NDM</sub> gene were used as donors, with the rifampicin-resistant *Escherichia coli* EC600 strain serving as the recipient. In brief, the donor and recipient strains were mixed in a ratio of 2:1 in LB broth, and the mixture was cultured overnight at 37°C. The cultures were then inoculated onto LB agar plates containing 1 µg/mL meropenem and 500 µg/mL rifampicin to select for successfully conjugated strains. To confirm the successful plasmid conjugation, the presumed conjugants were purified and subsequently validated using MALDI-TOF MS and PCR.<sup>13</sup>

## Multilocus Sequence Typing (MLST)

Following the primer provided by the pubMLST website(<https://pubmlst.org/ecloacae/>), PCR amplification was performed on seven housekeeping genes. The positive PCR products were then sent to Shenggong Biotechnology Co. Ltd. (Shanghai, China) for sanger sequencing. The obtained sequences are submitted to the pubMLST website to determine the sequence type (ST) for each isolated strain. For the sequences that cannot be matched accurately, upload the necessary information according to the requirements of the website and wait for the assignment of a new sequence number.

## Statistical Analysis

Data analysis was performed with SPSS v.26.0 software. In the assessment of risk factors, different statistical tests were employed based on the type of the data. Analysis of categorical variables utilizing either the chi-square test or Fisher's exact test. Continuous variables were assessed by utilizing either the Student's *t*-tests or Mann-Whitney *U*-test, the selection of which was contingent upon the normality assumption of the data distribution. In the univariate analysis, variables with  $P < 0.1$  were included in the multivariate logistic regression analysis to determine independent risk factors for CREC infection. A statistically significant result was defined as *P*-value less than 0.05 (two-tailed).

## Results

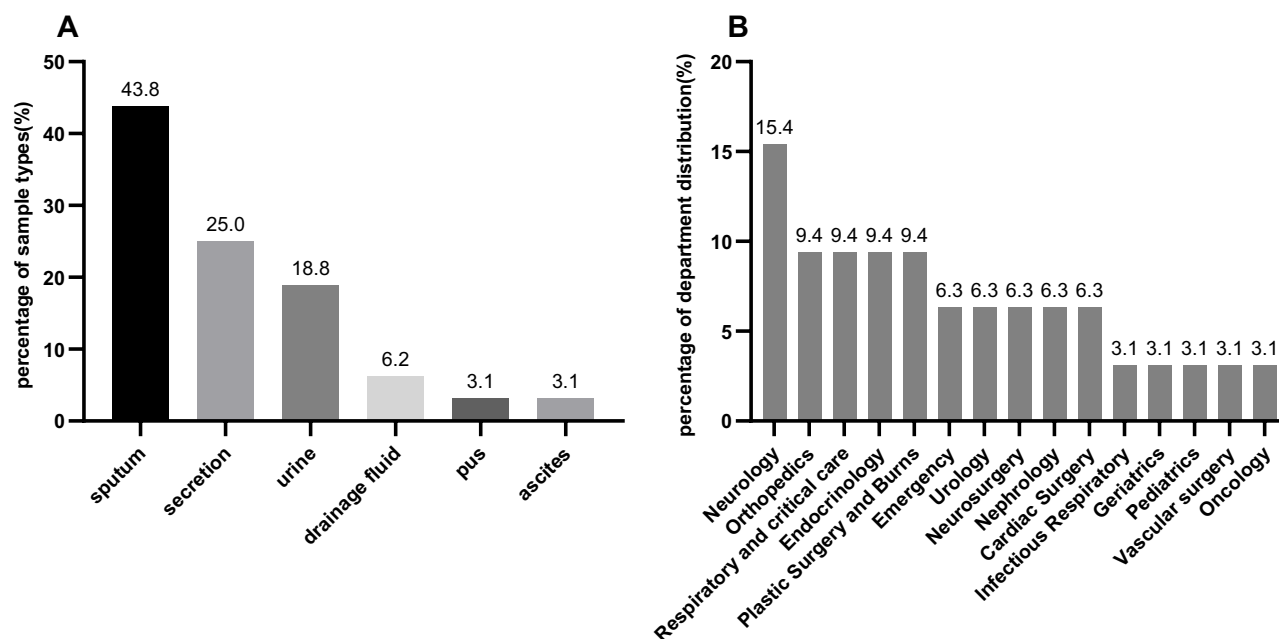
### Basic Characteristics of the CREC Strains

Between 2020 and 2023, a total of 32 non-duplicated CREC strains were isolated in our hospital. As shown in [Figure 1](#), they were obtained from different specimen types: sputum ( $n = 14$ , 43.8%), secretions ( $n = 8$ , 25%), urine ( $n = 6$ , 18.8%), drainage fluid ( $n = 2$ , 6.2%), pus ( $n = 1$ , 3.1%), and ascites ( $n = 1$ , 3.1%). Predominantly, departments such as burn surgery, orthopedics and neurology are the main sources. The majority of patients exhibited a positive outcome, with most achieving recovery or showing significant improvement. However, three cases ( $n = 3$ , 9.4%) resulted in death, suggesting that the CREC strains may be a low-virulence bacterium.

This study compiled data on CREC isolates from the Southwest Medical University affiliated hospital over the years 2020 to 2023. In 2020, there were six isolates, followed by three isolates in 2021, seventeen isolates in 2022, and two isolates in January and February 2023. A gradual increase in the rate of CREC isolation has been observed in recent years, remaining at approximately 2% prior to 2021 but surging to 6.8% in 2022. This significant and swift escalation in isolation rates merits substantial attention.

### Risk Factors of CREC Infections

In the analysis of risk factors, statistically significant differences were observed between the CREC group and the CSEC group in terms of cardiovascular diseases ( $P = 0.007$ ), respiratory system diseases ( $P = 0.039$ ), renal diseases ( $P = 0.009$ ), gastrointestinal diseases ( $P = 0.026$ ), invasive catheterization ( $P = 0.028$ ) and use of aminoglycosides ( $P = 0.041$ ) ([Table 1](#)). However, in the multivariate logistic regression analysis, no independent risk factors for CREC infections were identified.



**Figure 1** Distribution characteristics of 32 CREC isolates. (A) Sample types of 32 CREC isolates; (B) department distribution of 32 CREC isolates.

## Species Identification of 20 CREC Strains

Seven MLST alleles were combined with the *hsp60* gene, and a phylogenetic tree was constructed using Mega11.0 software with the Maximum Likelihood method. As shown in Figure 2, *Enterobacter hormaechei* subsp. *xiangfangensis* (n = 10) was the predominant strain type, followed by *Enterobacter hormaechei* subsp. *steigerwaltii* (n = 7), *Enterobacter kobei* (n = 2), and *Enterobacter hormaechei* subsp. *hoffmannii* (n = 1).

**Table 1** Clinical Characteristics of CREC and CSEC Strains

Variable	CREC(n=32)	CSEC(n=64)	P-value
Demographic, n (%) or IQR			
Age (≥60)	17(53.1%)	23(35.9%)	0.107
Sex (male)	22(68.8%)	43(67.2%)	0.877
Length of hospital stay	21(12, 34)	18(12, 33)	0.496
Admission to ICU	11(34.4%)	21(32.8%)	0.878
Co-morbidity, n (%)			
Malignant disease	4(12.5%)	13(20.3%)	0.345
Diabetes mellitus	9(28.1%)	16(25%)	0.742
Hypertension	15(46.9%)	25(39.1%)	0.464
Cardiovascular diseases	15(46.9%)	13 (20.3%)	<b>0.007</b>
Hepatobiliary disease	12(37.5%)	24(37.5%)	1.000
Respiratory disease	24(75%)	34(53.1%)	<b>0.039</b>
Renal disease	20(62.5%)	22(34.4%)	<b>0.009</b>

(Continued)

**Table 1** (Continued).

Variable	CREC(n=32)	CSEC(n=64)	P-value
Urinary tract infection	10(31.3%)	11(17.2%)	0.116
Craniocerebral disease	18(56.3%)	23(35.9%)	0.058
Gastrointestinal disease	14(43.8%)	14(21.9%)	<b>0.026</b>
Trauma and invasive procedures			
Severe trauma	5(15.6%)	20(31.3%)	0.100
Bronchoscope	3(9.3%)	7(10.9%)	1.000
Surgery	25(78.1%)	47(73.4%)	0.617
Invasive catheter	24(75%)	33(51.6%)	<b>0.028</b>
Antibiotic treatment, n (%)			
Penicillins	9(28.1%)	11(17.2%)	0.214
First, second-generation cephalosporins	3(9.4%)	8(12.5%)	0.747
Third, fourth-generation cephalosporins	11(34.4%)	23(35.9%)	0.880
Aminoglycosides	4(12.5%)	1(1.5%)	<b>0.041</b>
Quinolones	9(28.1%)	9(14.1%)	0.096
Carbapenems	4(12.5%)	7(10.9%)	1.000
Clinical outcomes, n (%)			
Patient outcome: mortality	3(9.4%)	3(4.7%)	0.397

**Note:** The bolded values indicate  $P < 0.05$ .

**Abbreviations:** CR, carbapenem-resistant; CS, carbapenem-susceptible; IQR, interquartile range; ICU, intensive care unit.

## Antimicrobial Susceptibility Tests

All CREC strains in this study were resistant to three or more antibiotics and were defined as MDR. According to the results of antimicrobial susceptibility tests, the most sensitive antibiotics were tigecycline (100%) and polymyxin B (100%), followed by amikacin (85%). All strains showed extreme resistance to a variety of antibiotics, with 100% resistance rates to imipenem, meropenem, cefazolin, cefuroxime, ceftriaxone, cefotaxime, ceftazidime and cefepime. The antibiotic susceptibility profile was shown in [Table 2](#).

## Phenotype and Genotype Analysis

As shown in [Figure 2](#). All 20 CREC strains tested positive for both mCIM and eCIM assays, indicating the production of carbapenemases. PCR experiments revealed that all 20 CREC strains carried the *bla*<sub>NDM</sub> gene. Upon further sequencing analysis, it was found that 13 strains carried the *bla*<sub>NDM-1</sub> gene (n = 13, 65%), while 7 strains carried the *bla*<sub>NDM-5</sub> gene (n = 7, 35%). No other resistance genes were detected, suggesting that *bla*<sub>NDM</sub> was the predominant gene in the local area for CREC. Conjugation experiments showed that 13 CREC strains were able to transfer the resistance plasmids to recipient strains. PCR confirmation revealed that all transconjugants were positive for the *bla*<sub>NDM</sub> gene.

Additionally, the most frequently detected ESBL and AmpC resistance gene was *bla*<sub>ACC</sub> (n = 17, 85%), followed by *bla*<sub>TEM</sub> (n = 11, 55%), *bla*<sub>SHV</sub> (n = 8, 40%), and *bla*<sub>CTX-M</sub> (n = 6, 30%).

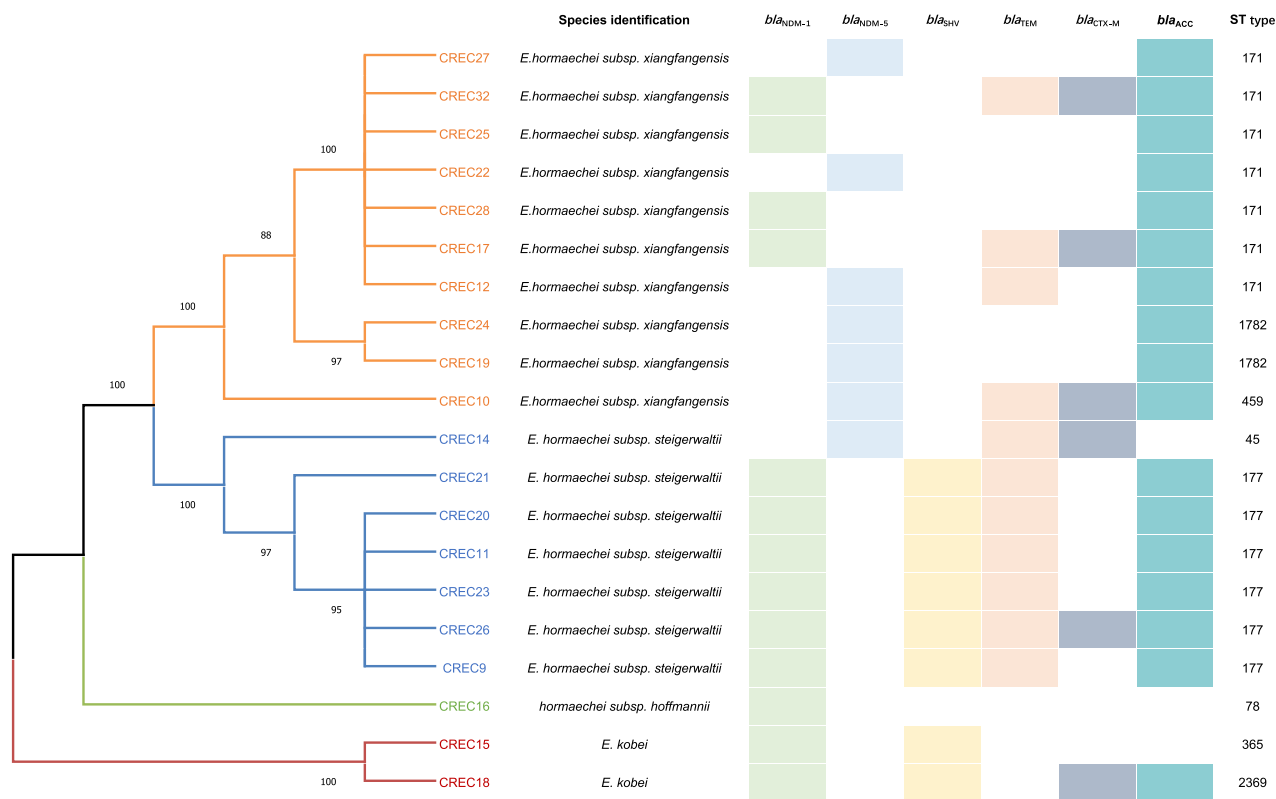


Figure 2 Species identification and clonal phylogenetic analysis of 20 strains of CREC.

### Multilocus Sequence Typing (MLST)

A total of eight ST types were detected, including seven known ST types and one novel ST type designated as ST2369, as assigned by the pubMLST official website. The most common ST types, apart from the novel ST2369, were ST171 (n = 7) and ST177 (n = 6). Other less frequent ST types included ST1782, ST45, ST78, ST365, and ST459. It is noteworthy that the recent clinical isolates predominantly belonged to the ST171 sequence type, indicating a possible recent dissemination of this clone within our hospital. It was recommended to implement relevant measures to address this situation.

Table 2 Antimicrobial Susceptibility Profiles of 20 Clinical CREC Strains

Antimicrobial Agent	NO.	%R	NO.	%I	NO.	%S
Amikacin	2	10	1	5	17	85
Imipenem	20	100	0	0	0	0
Meropenem	20	100	0	0	0	0
Cefazolin	20	100	0	0	0	0
Cefuroxime	20	100	0	0	0	0
Ceftriaxone	20	100	0	0	0	0
Cefotaxime	20	100	0	0	0	0
Ceftazidime	20	100	0	0	0	0
Cefepime	20	100	0	0	0	0
Sulperazon	19	95	1	5	0	0
Ciprofloxacin	18	90	0	0	2	10
Levofloxacin	14	70	2	10	4	20

(Continued)

Table 2 (Continued).

Antimicrobial Agent	NO.	%R	NO.	%I	NO.	%S
Tobramycin	10	50	6	30	4	20
Aztreonam	14	70	1	5	5	25
Ampicillin/sulbactam	20	100	0	0	0	0
Piperacillin/tazobactam	20	100	0	0	0	0
Cefoxitin	20	100	0	0	0	0
Tigecycline	0	0	0	0	20	100
Polymyxin B	0	0	0	0	20	100

Abbreviations: S, susceptible; I, intermediate; R, resistant.

## Comparison of Carbapenem Resistance Genes Carried by Different Genetic Strains

Among the 20 isolates in this study, *Enterobacter hormaechei subsp. xiangfangensis* (60%) was the main carrier of *bla*<sub>NDM-5</sub>, while other isolates mainly carried *bla*<sub>NDM-1</sub>. This indicated that there were differences in the types of carbapenemases carried by different subspecies of CREC strains. Additional sample sizes are required to investigate this phenomenon thoroughly.

## Discussion

The infection caused by CREC has been paid more and more attention in clinical settings. As an emerging and common pathogen, CREC strains posed a serious threat to antimicrobial therapy.<sup>14</sup> In this study, we conducted an analysis of CREC strains isolated from a teaching hospital in southwestern China. The results showed that the CREC strains exhibited high levels of resistance to most antibiotics. Molecular epidemiological studies revealed that ST171 and ST177 were the predominant sequence types, and *bla*<sub>NDM-1</sub> and *bla*<sub>NDM-5</sub> were the major antimicrobial resistance genes.

*Enterobacter cloacae* complex (ECC) includes multiple members.<sup>15</sup> Distinguishing between these species using traditional phenotypic methods was difficult, and there was still controversy regarding the nomenclature of each member.<sup>16</sup> For more precise molecular typing, Harald Hoffmann categorized ECC strains into 12 genetic clusters and one unstable sequence type based on the variations in the *hsp60* gene.<sup>3</sup> This classification system provided a more accurate approach to studying the classification, evolution, epidemiology, and pathogenicity characteristics of ECC strains. In this study, we classified the 20 CREC strains involved using the latest described method.<sup>2</sup> Our research discovered that *Enterobacter hormaechei* was the predominant strain, with *Enterobacter hormaechei subsp. xiangfangensis* being the major representative, which was consistent with the findings of the majority of other studies.<sup>17,18</sup>

This study, which began in December 2020, investigated the resistance mechanisms and clinical epidemiology of CREC isolates in our hospital. The results of antibiotic susceptibility testing showed a 100% resistance rate to imipenem and meropenem, indicating a MDR phenotype. Polymyxin B and tigecycline appear to be the most effective treatments for these clinical isolates. However, there have been recent widespread reports of tigecycline-resistant CREC isolates in China, which required sufficient attention.<sup>19,20</sup>

New Delhi metallo- $\beta$ -lactamase (NDM) belongs to the class B enzymes and is a type of metallo- $\beta$ -lactamases (MBLs).<sup>21</sup> In this study, the main mechanism of carbapenem resistance among the CREC strains was attributed to the production of NDM-1. And the *bla*<sub>NDM</sub> was identified as the only carbapenemase-encoding gene found in this study. Another research analyzing CREC strains from 11 regions in China confirmed that NDM was the predominant carbapenemase type, consistent with the findings of this study.<sup>22</sup> Since its discovery in India in 2008, NDM has rapidly spread worldwide.<sup>23,24</sup> Its flexible hydrolytic mechanism and increasing mutations have intensified the threat of antibiotic resistance. Extensive research has been undertaken with the objective of discovering potent inhibitors capable of reversing this prevailing condition.<sup>25,26</sup> It has been found that the majority of *bla*<sub>NDM</sub> was located on plasmids, and due to the transferability of plasmids, the horizontal spread of plasmid-mediated resistance genes may occur if strict isolation measures are not taken.<sup>27</sup> The plasmid conjugation transfer experiments in this study demonstrated that 13 CREC isolates were capable of transferring plasmids to EC600 recipient bacteria, conferring resistance and promoting



the development of multidrug-resistant bacteria. This finding aligns with a study conducted in other southwestern regions of China.<sup>28</sup> Plasmid-mediated horizontal gene transfer promotes the formation of the MDR phenotype in bacteria, further complicating the treatment of infections.

A total of eight sequence types were identified, with ST171 (n = 7) and ST177 (n = 6) being the most common. Previous research has examined the transmission of two different lineages of ST171 in the United States, highlighting ST171 as a high-risk clone with significant epidemic potential.<sup>29</sup> However, recent studies have indicated its widespread presence across various regions.<sup>30,31</sup> Another high-risk clone, ST78, was also identified in this study (n = 1), although it has not caused extensive outbreaks in the local region, appropriate measures should still be taken. When comparing different genetic clusters of bacteria, the *bla*<sub>NDM-5</sub> gene was found to be predominantly distributed in *Enterobacter hormaechei* subsp. *xiangfangensis*, showing differences from bacteria in other genetic clusters. This phenomenon warranted further investigation. Conversely, all *Enterobacter hormaechei* subsp. *steigerwaltii* isolates carried the *bla*<sub>NDM-1</sub> gene, which is significantly different from *Enterobacter hormaechei* subsp. *xiangfangensis*, suggesting variations in the enzyme types carried by different genetic clusters of bacteria. In this study, a new ST type, ST2369, was also identified. Through *hsp60* gene sequencing, it was determined to be *Enterobacter kobei* and carried the *bla*<sub>NDM-1</sub> gene. This is the first report of an ST2369 isolate of carbapenem-resistant *Enterobacter kobei* carrying the *bla*<sub>NDM-1</sub>. This newly discovered type requires further research to determine its specific characteristics and understand the spread of this clone. Its pathogenic potential should not be overlooked.

This study has some limitations. Firstly, the number of strains included in the study was relatively small, and it would be beneficial to collect more strains for future research. Secondly, the study was conducted in a single center, which limits its ability to represent the prevalence of strains in other regions. The results may have some bias due to the localized sample collection. It is important to expand the scale of the study in the future to conduct more comprehensive investigations and characterizations of CREC strains at a deeper level.

## Conclusion

In this study, we described the molecular characteristics, risk factors and resistance mechanism of CREC strains in a tertiary hospital in Sichuan, China. ST171 and ST177 were the main sequence types. NDM was the predominant carbapenemase discovered and can be disseminated through plasmids, which was also the main reason for carbapenem resistance of CREC strains in our hospital. A new ST (ST2369) strain carrying the *bla*<sub>NDM-1</sub> gene was detected. This research could help to better understand CREC strains and improve infection control and treatment in hospitals.

## Data Sharing Statement

The data used or analyzed in this study can be obtained from the corresponding author upon reasonable request.

## Ethics Approval and Consent to Participate

This study received approval from the Ethics Review Committee of the Affiliated Hospital of Southwest Medical University (KY2022267), in accordance with the principles outlined in the Declaration of Helsinki. Additionally, all patients participated by providing written informed consent.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; 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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