Original Article

Antibiotic susceptibility and molecular analyses of clinical *Enterobacter cloacae* isolates in Eastern Heilongjiang Province, China

Kewang Hu[1#], Lingyi Zeng[1#], Jisheng Zhang[2], Huiling Li[1], Shanshan Su[1], Yongxin Zhao[1], Yong Wang[1], Yanjun Fu[1], Chunjiang Li[3], Xiaoli Zhang[1,2]

[1]Department of Microbiology, First Affiliated Hospital of Jiamusi University, Jiamusi 154000, China; [2]Department of Microbiology, Yongchuan Hospital of Chongqing Medical University, Chongqing 402160, China; [3]Department of Pathogenic Biology, Jiamusi University School of Basic Medicine, Jiamusi 154007, China

Contributions: (I) Conception and design: X Zhang, C Li; (II) Administrative support: J Zhang, H Li; (III) Provision of study materials or patients: S Su, Y Fu; (IV) Collection and assembly of data: K Hu, L Zeng; (V) Data analysis and interpretation: Y Wang, Y Zhao; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Xiaoli Zhang. The First Affiliated Hospital of Jiamusi University, Department of Microbiology, No. 348, Dexiang Street, Jiamusi 154000, China. Email: jmszxl123@163.com; Chunjiang Li. Department of Pathogenic Biology, Jiamusi University School of Basic Medicine, No. 258, Xuefu Street, Jiamusi 154007, China. Email: 3355806796@qq.com.

Background: *Enterobacter cloacae* is an emerging opportunistic pathogen. We retrospectively conducted a study to assess antimicrobial susceptibility and investigated the Molecular characteristics of carbapenem-resistant *Enterobacter cloacae* (CREL) isolates.

Method: Three hundred forty-two isolates of *Enterobacter cloacae* were collected from January 2014 to December 2018. Ten strains of CREL were collected for further research. The species identifications and minimum inhibitory concentrations (MICs) of all antibiotics tested were analyzed using the Vitek 2 Compact system (BioMerieux, France) and supplemented by the disk diffusion method. Polymerase chain reaction (PCR) was performed to detect extended-spectrum β-lactamase (ESBL) and carbapenemase resistance genes.

Results: The results showed that most of the isolates remained susceptible to tested antibiotics; however, the resistance rate of Cefepime has been increasing in recent years. One strain co-producing New Delhi Metallo-β-lactamase NDM-1 and Imipenem hydrolase IMP-4. NDM-1 and IMP-4-producing isolates highlight that active surveillance is necessary to prevent the further spread of the bacteria. Multilocus sequence typing (MLST) showed that two KPC-producing isolates assigned to ST93, two isolates carrying NDM-1 assigned to ST1120. Moreover, the MEGA analysis showed that ST93, ST256, and ST1120 have homology, showing that CREL in our area has a potential spread risk.

Conclusions: These findings indicating that CREL clonal dissemination may occurred in this region and should be taken seriously concern. Our study highlights an urgent need to monitor these isolates to prevent their further spread.

Keywords: Enterobacter cloacae; antibiotic susceptibility; molecular analyses

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Introduction

Enterobacter cloacae are intrinsically resistant to ampicillin, amoxicillin-clavulanate, and first- and second-generation cephalosporins because producing chromosome mediated AmpC β-lactamase (1). In the case of low host immunity, dysbacteriosis, or other similar cases, respiratory, bloodstream, and surgical site infections are usually caused (2). The outbreak and spread of *Enterobacter cloacae* has been widely reported around the world (3), and even worse, because improper use of antibiotics, the spread of drug-resistant bacteria through various resources of the health care system, lack of appropriate guidelines for the use of antimicrobial agents, the resistance situation has gradually deteriorated. It has become a significant threat to global public health (4). The primary factors leading to β-lactam antimicrobials resistance of *Enterobacter cloacae* may be plasmid-mediated AmpC β-lactamases (pAmpC), plasmid-coded CTX-M family extended-spectrum β-lactamases, KPC family carbapenemases, and VIM, IMP and NDM-1 metallo-β-lactamases (5). In recent years, with the widespread use of Carbapenem antibiotics, the prevalence and spread of carbapenem-resistant Enterobacteriaceae (CRE) has become a global trend.

We conducted a retrospective study to evaluate the clinical distribution and antibiotic resistance of clinical *Enterobacter cloacae* isolates and the molecular analyses of CREL to promote the rational use of clinical antibiotics and to demonstrate the resistance mechanism of CREL in Eastern Heilongjiang Province, China.

Methods

Setting and isolates

The retrospective study was conducted at the First Affiliated Hospital of Jiamusi University with 1,800 beds in Eastern Heilongjiang Province, China. Three hundred forty-two strains of *Enterobacter cloacae* were collected from January 2014 to December 2018, all isolates were found at the species level, and routine antibiotic susceptibility tests were performed by the VITEK2 compact automated system. Unfortunately, we have been preserving CREL isolates regularly since 2016, so the earlier isolates were not preserved. Seven strains of CREL were collected from September 2016 to December 2018, and 3 added strains were collected in 2019. All of them were stored in a –80 ℃ refrigerator for further analysis.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all isolates was evaluated by the VITEK2 Compact automatic system AST GN card (bioMérieux, France), supplemented by a disk diffusion method. As CREL were considered only those isolates that were confirmed as carbapenems-nonsusceptible (either of ertapenem, imipenem or meropenem) according to Clinical and Laboratory Standard Institutes (CLSI-2016) criteria. E. coli ATCC 25922 was used as the control for antimicrobial susceptibility testing.

Detection of resistance genes

DNA was extracted from the bacteria using the boiling method. Polymerase chain reaction (PCR) and nucleotide sequencing techniques were performed in 10 CREL strains to confirm the existence of carbapenemase genes bla_{KPC}, bla_{NDM}, bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-4}, bla_{IMP-8}, bla_{OXA-1}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-51}, bla_{OXA-58}, and ESBL genes, including bla_{CTX}, bla_{TEM}, bla_{ACC}, and bla_{SHV}, using primers as previously described and listed at *Table 1* (6). According to the size of the target fragment, positive amplification products were sequenced, and the sequencing results were compared against the BLAST tool.

Multilocus sequence typing (MLST)

MLST was performed using seven housekeeping genes of *Enterobacter cloacae*, which were amplified using primers shown in online databases (https://pubmlst.org/ecloacae/). The products of PCR were sequenced. Sequence types (STs) were determined using online database tools. Molecular Phylogenetic analysis by Maximum Likelihood method, evolutionary analyses were conducted in MEGA X software.

Statistical analysis

The SPSS 25.0 software was conducted using the Chi-square test for statistical analysis. $P<0.05$ was statistically significant.

Results

Clinical data

From January 2014 to December 2018, a total of 342 non-repetitive Enterobacter cloacae were detected. The most

Table 1 Primers used in this study

Resistance gene	Primer sequence
KPC	F: TGTCACTGTATCGCCGTC
	R: CTCAGTGCTCTACAGAAAACC
NDM	F: GGGCAGTCGCTTCCAACGGT
	R: GTAGTGCTCAGTGTCGGCAT
VIM-1	F: AGTGGTGAGTATCCGACAG
	R: ATGAAAGTGCGTGGAGAC
VIM-2	F: ATGTTCAAACTTTTGAGTAAG
	R: CTACTCAACGACTGAGCG
IMP	F: ACCGCAGCAGAGTCTTTGCC
	R: ACAACCAGTTTTGCCTTACC
OXA-23	F: GATCGGATTGGAGAACCAGA
	R: ATTTCTGACCGCATTTCCAT
OXA-24	F: GGTTAGTTGGCCCCCTTAAA
	R: AGTTGAGCGAAAAGGGGATT
OXA-48	F: TTGGTGGCATCGATTATCGG
	R: GAGCACTTCTTTTGTGATGGC
OXA-51	F: TAATGCTTTGATCGGCCTTG
	R: TGGATTGCACTTCATCTTGG
OXA-58	F: AAGTATTGGGGCTTGTGCTG
	R: CCCCTCTGCGCTCTACATAC
DHA	F: AACTTTCACAGGTGTGCTGGGT
	R: CCGTACGCATACTGGCTTTGC
ACC	F: AACAGCCTCAGCAGCCGGTTA
	R: TTCGCCGCAATCATCCCTAGC
SHV	F: CTTTACTCGCCTTTATCGGC
	R: TTACCGACCGGCATCTTTCC
TEM	F: GTGCGCGGAACCCCTATT
	R: TTACCAATGCTTAATCAGTGAGGC
CTX-M-1	F: GGTTTAAAAAATCACTGCGTC
	R: TTGGTGACGATTTTAGCCGC
CTX-M-9	F: ATGGTGACAAAGAGAGTGCA
	R: CCCTTCGGCGATGATTCTC

Table 2 Specimens type and department distribution of *Enterobacter cloacae*

Specimen distribution	No.
Clinical departments	
Respiratory medicine	48
Neurology	42
Emergency ICU	28
Orthopaedics	27
Emergency department	26
Pediatrics	23
Burns surgery	19
General surgery	14
Infectious diseases	14
Cardiology	12
Urology	10
Critical care medicine	10
Nephrology	9
Others	60
Specimen type	
Sputum	202
Secretion	41
Blood	39
Pus	18
Urine	13
Drainage fluid	3
Others	26

No., strain number.

common specimens were sputum (202/342, 59%), followed by secretion (41/342, 12%), blood (39/342, 11.4%). The most common clinical departments were respiratory, followed by neurosurgery, emergency ICU, orthopedics, and emergency department (*Table 2*). CREL information was shown in *Tables 3* and *4*. The trends of resistance rate of *Enterobacter cloacae* were shown in *Figure 1*.

Table 3 Microbiological and molecular characteristics of carbapenem-resistant *Enterobacter cloacae* strains.

NO.	Separation date	Gender	Age	Department	Specimen type	Antibiotic sensitivity test results												
						IMP	MEM	ETP	AMP	SAM	TZP	CFZ	CAZ	CRO	FEP	AMK	LEV	SXT
1	2016.9.6	Male	31	Emergency surgery	Secretions	R	R	R	R	R	R	R	R	R	R	S	S	S
2	2016.9.23	Female	59	Emergency ICU	Blood	I	R	R	R	R	S	R	R	R	I	R	S	R
3	2017.7.16	Male	83	Infectious diseases	Urine	R	R	R	R	R	R	R	R	R	R	S	R	R
4	2017.7.13	Female	82	Emergency ICU	Pus	R	R	R	R	R	R	R	R	R	R	S	R	R
5	2017.9.22	Female	46	Emergency department	Blood	R	R	R	R	R	S	R	R	R	I	S	S	R
6	2018.3.19	Male	16	Emergency department	Blood	R	R	R	R	R	R	R	R	R	R	S	R	S
7	2018.5.15	Female	65	Neurology	Sputum	R	R	R	R	R	R	R	R	R	R	R	R	R
8	2019.05.08	Male	47	ICU	Sputum	R	R	R	R	R	R	R	R	R	R	S	S	R
9	2019.06.11	Female	29	Burn ward	Secretions	R	R	R	R	R	R	R	R	R	R	S	R	R
10	2019.07.29	Male	48	Emergency ICU	Blood	R	R	R	R	R	R	R	R	R	R	S	R	R

NO., strain number; IMP, imipenem; MEM, meropenem; ETP, ertapenem; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin/tazobactam; CFZ, cefazolin; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMK, amikacin; LEV, levofloxacin; SXT, sulfamethoxazole-trimethoprim; R, resistance; I, intermediate; S, sensitive.

Table 4 Molecular characteristics of carbapenem-resistant *Enterobacter cloacae* strains

NO.	Resistance gene													ST
	KPC-2	*NDM-1*	*IMP-4*	*VIM-1*	*VIM-2*	*SHV*	*ACC*	*DHA*	*TEM*	*CTX-M-1*	*CTX-M-9*	*OXA-1*	*OXA-48*	
1	–	+	–	–	–	+	–	–	–	+	–	+	–	1,119
2	–	–	+	–	–	–	–	–	+	–	–	+	–	520
3	–	+	+	–	–	+	–	–	+	+	–	–	–	1,120
4	+	–	+	–	–	–	–	+	+	–	–	+	–	93
5	–	–	+	–	–	+	–	+	+	–	–	–	–	528
6	+	–	–	–	–	+	+	+	+	+	+	+	–	93
7	–	+	–	–	–	+	–	–	+	+	–	–	–	1,120
8	–	–	–	–	–	+	–	+	+	–	–	–	–	528
9	–	–	–	–	–	+	–	–	–	+	+	+	–	171
10	–	–	–	–	–	+	+	+	+	–	–	+	–	256

NO., strain number; –, negative; +, positive; ST, sequence type.

Antimicrobial susceptibility test

As shown in *Table 5*, amikacin was the lowest in the five years, and the resistance rate of Enterobacter cloacae to ceftriaxone was the highest. The resistance rate of the fourth-generation cephalosporin cefepime has gradually increased since 2015. Both Piperacillin/Tazobactam and Cefepime have statistically difference in the resistance rates in the five years. Moreover, compared with non-CREL, the resistance rate of CREL strains was significantly higher ($P<0.05$)

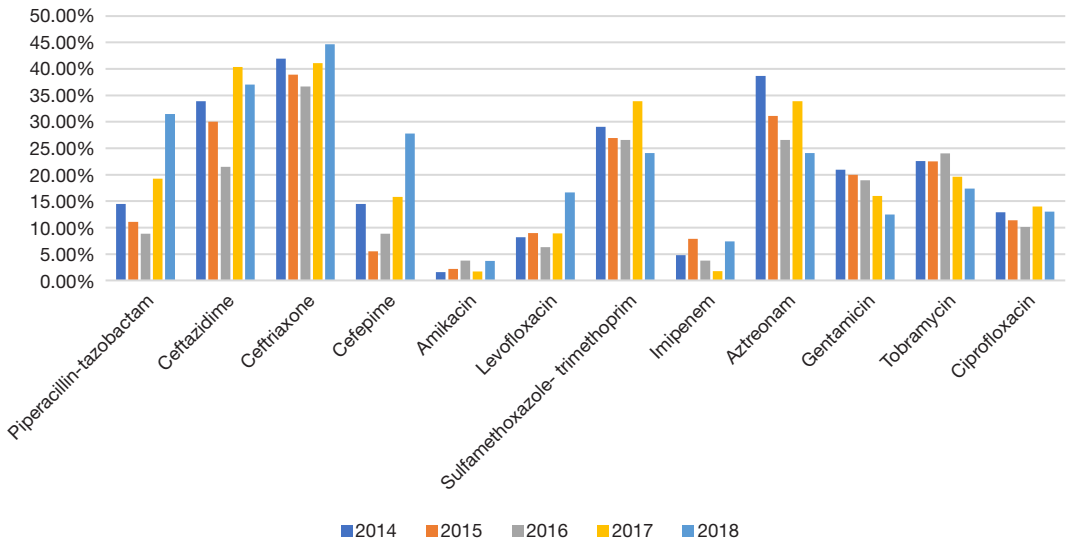

Figure 1 The trends of the resistance rate of *Enterobacter cloacae*.

Table 5 Resistance rates and statistical results of *Enterobacter cloacae* isolates to antimicrobial agents and comparison of CREL and non-CREL resistance

Antibiotics	2014		2015		2016		2017		2018		P	CREL		*non-CREL*		P
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%		S	N-S	S	N-S	
TZP	9/62	14.5	10/90	11.1	7/79	8.8	11/57	19.3	17/54	31.5	0.005	8	11	280	43	0.000
CAZ	21/62	33.9	27/90	30.0	17/79	21.5	23/57	40.4	20/54	37.0	0.151	5	14	241	82	0.000
CRO	26/62	41.9	35/90	38.9	29/79	36.7	24/57	42.1	24/54	44.4	0.917	4	14	196	120	0.001
FEP	9/62	14.5	5/90	5.5	7/79	8.8	9/57	15.8	15/54	27.8	0.003	6	13	291	32	0.000
AMK	1/62	1.6	2/90	2.2	3/79	3.8	1/57	1.7	2/54	3.7	0.887	16	3	317	6	0.010
LEV	5/62	8.2	8/90	8.9	5/79	6.3	5/57	8.7	9/54	16.6	0.314	8	11	299	21	0.000
SXT	18/62	29.0	24/90	26.7	21/79	26.6	19/57	33.3	13/54	24.0	0.818	9	10	236	85	0.019
ATM	24/62	38.7	28/90	31.1	21/79	26.6	19/57	33.3	21/54	38.9	0.575	3	13	204	86	0.000
GEN	13/62	20.9	18/90	20.0	15/79	19.0	9/57	15.7	6/54	11.1	0.919	8	7	225	47	0.011
TOB	14/62	22.6	20/90	22.5	19/79	24.0	11/57	19.2	9/54	17.4	0.955	8	8	222	57	0.011
CIP	8/62	12.9	10/90	11.1	8/79	10.1	8/57	14.0	7/54	13.0	0.968	6	10	252	25	0.000

n, number of resistant isolates; N, total number of isolates. S, susceptible; N-S: non-susceptible; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMK, amikacin; LEV, levofloxacin; SXT, sulfamethoxazole-trimethoprim; ATM, aztreonam; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin.

Molecular analyses

Among the ten strains, three types of carbapenemases were confirmed, including IMP-4, KPC-2, NDM-1. The corresponding numbers of the strains that produced the primary types of carbapenemases were 4, 3, 2. Other carbapenemase genes were not detected, with one strain co-producing NDM-1 and IMP-4. TEM and SHV were the most often ESBL gene, followed by OXA-1.

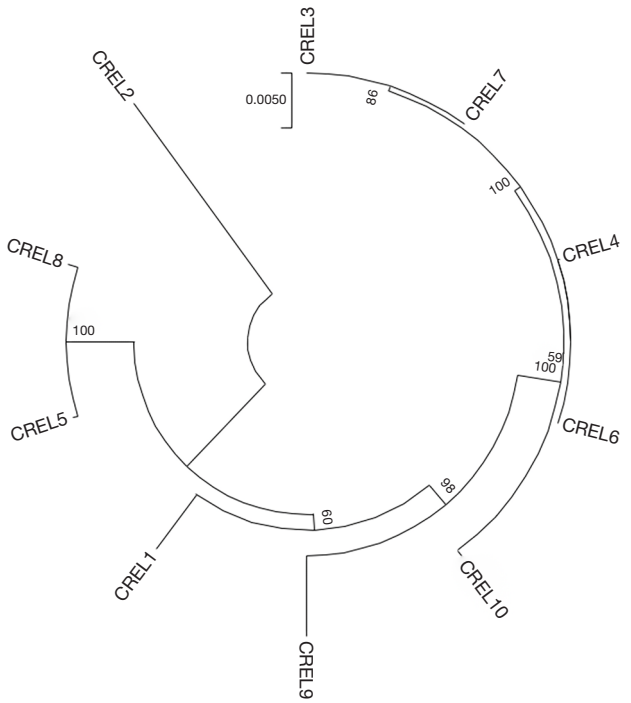

Figure 2 Molecular phylogenetic analysis of CREL. CREL, carbapenem-resistant *Enterobacter cloacae*.

MLST

The results of the MLST are shown in *Table 3*. A total of 7 sequence types were detected in the 10 CREL strains. ST93 (2/10), ST528 (2/10), and ST1120 (2/10) were isolated two of each ST, while the others were assigned to an isolate per ST. The phylogenetic tree built from the sequence data of the seven MLST genes showed that ST93, ST1120, ST256 has high homology (ST256 and ST1120 differed from ST93 by one and two housekeeping genes, respectively). The phylogenetic tree, as shown in *Figure 2*.

Discussion

Enterobacter cloacae is an important opportunistic pathogen and frequently implicated in nosocomial infections (7). The carrying of drug-resistant genes also leads to the multidrug-resistance and limiting therapeutic options even further (8). Our study showed that most of the isolates remained susceptible to tested antibiotics; however, CREL holds a significantly higher resistance rate compared with non-CREL. The drug resistance rate of Cefepime has been increasing in recent years, from 5.5% in 2015 to 27.8% in 2018. Previous research has reported that the combination of different resistance mechanisms, such as porin loss (primarily OmpC), expression of bla_{OXA-1}, and/or TEM-1, could confer decreased susceptibility to Cefepime (9-11). Cefepime resistance has been described in Pseudomonas aeruginosa and E.col strains producing OXA-1 beta-lactamase (12,13). In our study, 6 of 10 strains carrying the blaOXA-1 gene, which may be one of the reasons for the increased resistance rate of Cefepime in recent years. However, we only investigated CREL strains, the carrying rate of the blaOXA-1 gene in non-CREL strains, and other mechanisms that need further exploration.

Carbapenemase spread has been increasingly reported worldwide over the last decade (14). VIM-1 was most common in Spain and other southern European countries (15), in the current study, no producers of VIM-1 were found. A previous multicenter study conducted a molecular epidemiological survey of carbapenem-resistant *Enterobacter cloacae* (CREL) in 11 cities in China to understand the prevalence of the bacteria further. E. cloacae prevalent in China produced NDM-1 and IMP-4. NDM-1 was found in the highest proportion and may represent a significant drug-resistant mechanism of carbapenem-producing Enterobacteriaceae in China (1). In the present study, a higher proportion (4/10, 40%) of bla_{IMP-4} positive strains were identified among carbapenem-resistant E. cloacae isolates showing that IMP-4 was the dominant carbapenemase instead of NDM-1 in this region. Australia has observed the dominance of IMP-4-producing *Enterobacter cloacae*, which agreed with the current study (16). It is worth noting that one strain of this study co-producing NDM-1 and IMP-4. One CREL strain was previously reported to co-producing NDM-1 and IMP-8 in Chongqing, China (17). NDM and IMP types belong to class B metallo-lactamases (MBLs) in the Ambler classification and disseminated among bacteria internationally (18). Considering that one strain co-producing NDM-1 and IMP-4 may confer multiple resistance to antibiotics that further reduce the therapeutic choices, surveillance for carbapenemase detection, and infection control measures should be implemented to prevent their further spread. The OXA-48 carbapenemase was first discovered in various Enterobacteriaceae species isolated in Turkey and other countries in the Middle East, also be reported in Enterobacter cloacae (19). It belongs to the D-class carbapenemase of Ambler classification, which has a weak role in the hydrolysis of carbapenems. OXA-48 was not detected in our study, showing that it is not

prevalent in our region. TEM and SHV was the dominant ESBL type in our study, unlike CTX-M-producers most frequent in Latin American countries (20).

MLST showed subtype diversity. Our study has revealed that local carbapenem-resistant E. cloacae isolates did not evolve from a unique ancestral background. A total of seven sequence types were detected in 10 carbapenem-resistant E. cloacae strains. Previous studies have reported some sporadic cases of E. cloacae isolates harboring NDM-1, such as ST92 in Croatia, ST265 in Australia (21,22). Our study identified a potential prevalent clone of ST1120 carbapenem-resistant E. cloacae isolates carrying NDM-1. However, this ST was different from some widespread E. cloacae STs (ST66, ST78, ST108, and ST114) that reported in European countries (23). The primary epidemic strains in Shenzhen city and Henan province in china were ST418 and ST120, respectively (1,24). The outbreak of ST88 carbapenem-resistant E. cloacae isolates carrying NDM-1 was reported in Chongqing, China (17). The first IMP-4-Producing Enterobacter cloacae sequence type 74 and 194 have reported in Korea in 2017 (25). Our results showed that four strains of IMP-4-producing assigned different ST typing revealed the genetic diversity of carbapenem-resistant E. cloacae. KPC carbapenemase is primarily found in Klebsiella pneumoniae, while KPC-producing E. cloacae infections have been relatively infrequent (26). However, the outbreak of KPC-producing E. cloacae ST114 has been reported in the United States (27). In China, the first KPC-producing CREL appeared in Shanghai in 2010 (28). In the present study, two KPC-producing isolates are assigned to ST93. Moreover, the MEGA analysis showed that ST93, ST256, and ST1120 have high homology, showing that CREL in our area has a potential spread risk. Since limited numbers were collected, the NDM-1-possessing ST1120 isolates, KPC-producing ST93 strains, and IMP-4-producing strains in our region should be taken with serious concern and still need to be further monitored.

In conclusion, our study investigated antibiotic susceptibility and molecular analyses of clinical Enterobacter cloacae isolated in Eastern Heilongjiang Province, China. The results showed that antimicrobial resistance was low among them. Compared with non-CREL, the resistance rate of CREL strains was significantly higher. ST93, ST1120, ST256 has high homology showing that CREL in our area has potential spread risk. The NDM-1-possessing ST1120 isolates, KPC-producing ST93 strains, and IMP-4-producing strains in our region should be taken with serious concern s and still need to be further monitored.

There are some limitations in this study: (I) we did not determine the exact mechanism of how each E. cloacae acquired the carbapenemase. Further studies are necessary to determine the exact mechanisms by which E. cloacae acquired the carbapenemase. (II) The number of CREL collected was limited, and we only investigated one hospital, so our conclusion may not be comprehensive enough or extended directly to the whole region.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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