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ORIGINAL RESEARCH

Molecular Mechanisms and Epidemiology of Carbapenem-Resistant *Enterobacter cloacae* Complex Isolated from Chinese Patients During 2004–2018

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Background: The emergence and spread of carbapenem-resistant *Enterobacter cloacae* complex (ECC) have posed a serious threat to human health worldwide. This study aimed to investigate the molecular mechanism of carbapenem resistance and its prevalence among ECC in China.

Methods: A total of 1314 ECC clinical isolates were collected from the First Affiliated Hospital of Wenzhou Medical University from 2004 to 2018. Sensitivity to antibiotics was determined using the agar dilution method. The production of carbapenemases and the prevalence of resistance-associated genes were investigated using PCR. The expression of outer membrane porin (OMP) genes (*ompC*/*ompF*) and cephalosporinase gene *ampC* was analyzed by quantitative real-time PCR. The effect of efflux pump mechanism on carbapenem resistance was tested. ECC was typed by multilocus sequence typing (MLST).

Results: In this study, 113 carbapenem-nonsusceptible ECC strains were identified. The prevalence rates of carbapenemase genes $bla_{\text{KPC-2}}$ and bla_{NDM} were 12.4% (14/113) and 17.7% (20/113), and that of the extended-spectrum β-lactamase (ESBL) genes $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} were 28.3% (32/113), 27.4% (31/113), and 14.2% (16/113), respectively. Among 67 carbapenem-nonsusceptible ECC isolates producing non-carbapenemase, low expression of *ompC*/*ompF* and overexpression of *ampC* were found in 46 and 40 strains, respectively. In addition, the carbapenem resistance was related to the overexpression of the efflux pump in the study. Finally, the 113 carbapenem-nonsusceptible ECC strains were categorized into 39 different sequence types using MLST.

Conclusion: Carbapenem-nonsusceptible ECC strains producing non-carbapenemase were predominant. The low expression of OMP with the overexpression of cephalosporinase or production of ESBLs and overexpression of efflux pump might contribute to the resistance to carbapenem for carbapenem-nonsusceptible ECC strains producing non-carbapenemase. The bla_{NDM} and bla_{KPC} comprised the principal resistance mechanism of carbapenemase-producing ECC in the hospital, causing a threat to public health. Therefore, monitoring programs to prevent the emergence and further spread of antibiotic resistance are urgently needed.

Keywords: carbapenemase, carbapenem-resistant mechanism, *Enterobacter cloacae* complex, epidemiology, non-carbapenemase

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Introduction

The *Enterobacter cloacae* complex (ECC) is one of the common microorganisms isolated in clinical specimens causing all kinds of infections, like pneumonia, urinary tract infections, and sepsis, in the last few decades.[1,2] Multidrug-resistant (MDR) ECC isolates have emerged and spread worldwide with the widespread use of antibiotics.[3] With the increase in resistance rates to aminoglycosides,

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fluoroquinolones, and third-generation cephalosporins, carbapenems, as the last-resort antibiotic, have gradually been used for treating MDR ECC infections.[1,4,5] However, the increasing resistance rates of carbapenems have gained special clinical attention.[6–8]

The mechanisms of carbapenem resistance in ECC are realized by either the acquisition of plasmid-encoded carbapenemase genes and the overexpression of efflux pumps, or, more commonly, the constitutive overexpression of AmpC or production of extended-spectrum β-lactamase (ESBL) combined with disrupted membrane permeability (the decrease in or loss of the outer membrane protein).[3,9] ECC is inherently resistant to first- and second-generation cephalosporins because the overexpression of an inducible AmpC β-lactamase is encoded by the chromosome gene *ampC*.[3,10] Moreover, the acquisition of plasmid-mediated ESBL genes, such as bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and so forth, makes ECC resistant to most β-lactam drugs, thus increasing the difficulty in clinical treatment.[11–15] Two major categories of carbapenem enzymes, carbapenem-hydrolyzing serine β-lactamases and metallo-β-lactamases, such as KPC, NmcA, IMI, FRI, GES, OXA, VIM, IMP, and NDM, have been identified in carbapenem-resistant Enterobacteriaceae.[3] The most common description of KPC and NDM-1 was in ECC isolates.[16]

Considering the increasing prevalence of carbapenem-nonsusceptible ECC isolates worldwide, longitudinal epidemiological surveillance and resistance mechanism study on the carbapenem-nonsusceptible ECC should be performed to control and prevent the distribution and spread of resistance, which is key to clinical significance in guiding antimicrobial therapy. However, relevant data on the long-term evolution of carbapenem-nonsusceptible ECC are lacking in China. In this study, the epidemiology prevalence and the molecular mechanisms of 113 ECC clinical isolates were characterized for carbapenem resistance during large-scale surveillance in the southeast of China. This study was novel in reporting ECC nonsusceptible to carbapenem antibiotics on a large scale in China.

Materials and Methods

Bacterial Isolates

A total of 1314 ECC clinical isolates were collected from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) from 2004 to 2018. Antimicrobial susceptibility testing and identification of all isolates were performed using a VITEK ®2 system (bioMérieux, Marcy-l'Étoile, France). The isolates were stored in 30% glycerol at –80°C prior to further analysis. All investigation protocols in this study were approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University. Informed consent was waived because this study with observational nature focused mainly on bacteria and involved no interventions to patients.

Minimum Inhibitory Concentration Determination

According to the latest guidelines recommended by the Institute of Clinical and Laboratory Standards (CLSI, 2020), the minimum inhibitory concentrations (MICs) of 15 antimicrobial agents, including aztreonam (ATM), ciprofloxacin (CIP), levofloxacin (LVX), ceftriaxone (CRO), cefepime (FEP), ceftazidime (CAZ), meropenem (MEM), imipenem (IPM), ertapenem (ETP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), trimethoprim-sulfamethoxazole (SXT), nitrofurantoin (NIT), and colistin (COL), were determined by the agar dilution method. Briefly, the bacteria were suspended in saline to one-tenth the turbidity of the 0.5 McFarland standard. The bacterial suspension was inoculated on the Mueller–Hinton (MH) agar plate containing different drug concentrations using the nail plate. The results were quantified by observing bacterial growth after incubation at 37°C for 16–20 h.[17] The MIC determination of colistin was explained by the recommendation of the European Committee on Antimicrobial Susceptibility Testing. *Escherichia coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility testing. All experiments were performed in triplicate.

Detection of Extended-Spectrum Beta-Lactamases

The American Clinical Laboratory Standardization Institute (CLSI) recommended ESBL confirmation test was performed.[1] Briefly, a lawn of test bacteria suspension equivalent to 0.5 McFarland turbidity standard solution was swabbed on the surfaces of MHA plates, and then ceftazidime (30 μg) and cefotaxime (30 μg) disks (Kanvax, China) with and without clavulanic acid (10 μg) were seeded within 15 min. All plates were then incubated aerobically at 37°C for 18 h. An isolate was phenotypically confirmed as an ESBL producer when a zone diameter difference of ≥5 mm was observed between both antibiotic disks with clavulanic acid and a similar agent without clavulanic acid.[18] The *E. coli* ATCC 25922 strain was

used as the negative control, and the *Klebsiella pneumoniae* ATCC 700603 strain was used as the positive control.

Detection of Antibiotic Resistance Determinants

The beta-lactamase genes, including carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{SPM}, bla_{IMI}, bla_{VIM}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-58}, bla_{Nmc-A}, bla_{FRI-1}, bla_{SME}, bla_{GIM}, bla_{BIC}, bla_{DIM}, bla_{AIM}, bla_{GES}, and bla_{SIM}) and extended-spectrum β-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-14}$), were identified by polymerase chain reaction (PCR). The primers of all genes are summarized in Table S1. The positive products of PCR amplification were sequenced by Shanghai Genomics Institute Technology Co. Ltd. (Shanghai, China). All sequencing results of the products were analyzed using BLAST searches against the NCBI database (www.ncbi.nlm.nih.gov/BLAST).

Efflux Pump Inhibition Assay

The efflux pump activity of carbapenem-insensitive ECC strains was determined using the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma, MO, USA). The MICs of ertapenem in the presence or absence of 8 μg/mL CCCP, which did not inhibit the growth of ECC bacteria, were determined by the agar dilution method. In the presence of CCCP, a reduction in carbapenem MIC ≥4 times was defined as the positive phenotype of the efflux pump downregulation.

Quantitative Real-Time PCR for Cephalosporinase AmpC and Outer Membrane Protein

The total RNA of 67 noncarbapenemase-producing ECC isolates was extracted. Then, 500 ng RNA was mixed with the reverse transcription system, and 10 μL of cDNA was obtained using a PrimeScript™ RT Kit (TaKaRa, Japan). Using a CFX-96 touch real-time PCR system, qPCR (Bio-Rad, CA, USA) was performed. Then, 100 ng cDNA, TB Green Premix Ex Taq II (Tli RNaseH Plus) (2×) (TaKaRa), and specific primers (*ompC* f: 5′-GCGACCAGACCTACATGCGT-3′, r: 5′-TTCGTTCTCACCAGAGTTACCCT-3′, *ompF* f: 5′-TCCC TGCCCTGCTGGTAG-3′, r: 5′-TAAGTGTTGTCGCCAT CGTTG-3′, *ampC* f: 5′-GCATGGCGGTGGCCGTTAT-′ r: 5′-CTGCTTGCCCGTCAGCTGT-3′) were added to each sample. The cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The expression levels of outer membrane genes *ompC* and *ompF* and cephalosporinase gene *ampC* were detected by RT-qPCR; the *rpoB* gene was used as the internal gene. Compared with carbapenem-sensitive *Enterobacter cloacae* ATCC700323, the target genes were quantified using the comparative threshold cycle $2^{-\Delta\Delta Ct}$ method. All experiments were repeated three times independently and averaged in the calculation of relative expression levels.

Multilocus Sequence Typing

The carbapenem-nonsusceptible ECC isolates were analyzed by amplifying seven housekeeping genes (*dnaA, fusA, gyrB, leuS, pyrG, rplB*, and *rpoB*). Sequence types (STs) were assigned by querying against the database available at the Institut Pasteur's *Enterobacter cloacae* MLST website (https://pubmlst.org/bigsdb?db=pubmlst_ecloacae_seqdef). Following the genetic similarity diagram using the goeBURST program, the clonal complexes were analyzed to identify the molecular epidemiological relationships.

Results

Bacterial Isolates and Antimicrobial Susceptibility Test

A total of 113/1314 (8.6%) carbapenem-nonsusceptible ECC isolates were determined with imipenem, meropenem, and ertapenem; 99 (7.5%) carbapenem-resistant strains were determined. The carbapenem-resistant ECC isolate was first detected in the hospital in 2010; the resistance rate increased from 2.5% in 2010 to 11.9% in 2018 (Table 1). The results of the antimicrobial susceptibility test of all 113 carbapenem-nonsusceptible isolates are listed in Table 2, which suggested higher resistance to fluoroquinolones, cephalosporins, and monobactams. Further, 63 (55.8%) and 45 (39.8%) ECC isolates were resistant to ciprofloxacin and levofloxacin, respectively; 30 (26.5%) and 35 (31.0%) isolates were resistant to gentamicin and tobramycin, respectively. Of note, 112 (99%) ECC strains were resistant to ceftriaxone. Nevertheless, 7 (6.2%) and 20 (17.7%) isolates were resistant to amikacin and colistin, respectively.

Frequency of β-Lactamase Genes

A total of 46 carbapenem-nonsusceptible ECC isolates carried carbapenemase genes (Figure 1). The prevalence rate of bla_{KPC-2}, bla_{NDM}, bla_{IMP}, and bla_{OXA-23} in carbapenem-nonsusceptible strains was 12.4% (14/113), 17.7%

Table 1 Carbapenem Susceptibility of ECC Clinical Isolates

Time of Isolation	No. of Isolates	CNS (R +I) (n)	NS (%)	R (%)	I (%)	S (%)
2004	32	0	0	0	0	100
2005	46	0	0	0	0	100
2006	47	0	0	0	0	100
2007	47	0	0	0	0	100
2008	60	0	0	0	0	100
2009	41	0	0	0	0	100
2010	40	1(1+0)	2.5	2.5	0	97.5
2011	34	5(4+1)	14.7	11.8	2.9	85.3
2012	82	6(5+1)	7.3	6.1	1.2	92.7
2013	36	1(1+0)	2.8	2.8	0	97.2
2014	36	4(4+0)	11.1	11.1	0	88.9
2015	177	20(19+1)	11.3	10.7	0.6	88.7
2016	214	31(25+6)	14.5	11.7	2.8	85.5
2017	221	20(16+4)	9.0	7.2	1.8	91.0
2018	201	25(24+1)	12.4	11.9	0.5	87.6
Total	1314	113(99+14)	8.6	7.5	1.1	91.4

Abbreviations: ECC, *Enterobacter cloacae* complex; No., number; CNS, carbapenem-nonsusceptible strains; S, sensitivity; I, intermediate; R, resistance; NS; R and I.

(20/113), 8.0% (9/113), and 3.5% (4/113), respectively, including one isolate carrying $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ specially (Figure 1), while $bla_{\text{Nmc-A}}$, bla_{BIC}, bla_{GES}, bla_{AIM}, bla_{GIM}, bla_{DIM}, bla_{SIM}, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-48}}$, $bla_{\text{FRI-1}}$, $bla_{\text{OXA-58}}$, bla_{SME}, and bla_{AIM} were not detected in all isolates. Moreover, 49 ECC strains were positive for the ESBL phenotypic test in 113 strains, and the ESBLs genes bla_{TEM} (27.4%, 31/113), $bla_{\text{CTX-M-14}}$ (19.5%, 22/113), $bla_{\text{CTX-M-1}}$ (15.9%, 18/113), $bla_{\text{CTX-M-9}}$ (14.2%, 16/113), and bla_{SHV} (14.2%, 16/113) among analyzed strains were also determined. In general, the harboring of carbapenemase genes and ESBLs accounted for 40.7% (46/113) and 58.4% (66/113), respectively.

Overexpression of the Efflux Pump

The efflux pump inhibition test was performed to explore the effect of the efflux pump on carbapenem resistance. The results showed that 36 strains had a ≥4 times reduction in ertapenem MICs in the presence of 8 μg/mL CCCP, suggesting that the efflux pump had a significant effect on carbapenem resistance.

Outer Membrane Protein Gene Expressions

This study investigated the relationship between resistance to carbapenems and the expression level of outer membrane genes *ompC* and *ompF* and cephalosporinase gene *ampC*. Further, 67 ECC noncarbapenemase-producing isolates and *Enterobacter cloacae* ATCC700323 as the control strain were used. The results of RT-qPCR showed that the expression level of *ompC* in 29 ECC isolates decreased compared with that in ATCC 700323, and the decreased expression of *ompF* was found in 42 ECC strains. In addition, 24 ECC isolates had low expression of both *ompC* and *ompF*. The overexpression of cephalosporinase gene *ampC* was found in 40 ECC strains, with the highest level as 26-fold (Figures 1 and S1). Further, 24 ECC isolates had the overexpression of cephalosporinase gene *ampC* and low expression of *ompC* or/and *ompF*. ESBLs and low-level expression of *ompC* or/and *ompF* were detected in 33 ECC isolates (Figure 1).

Multilocus Sequence Typing Analysis

The 113 carbapenem-nonsusceptible ECC isolates were assigned to 39 different STs, including the most prevalent ST190 (14.2%, 16/113), followed by ST114 (4.4%, 5/113), ST93 (4.4%, 5/113), ST97 (3.5%, 4/113), ST78 (3.5%, 4/113), ST32 (2.7%, 3/113), ST46 (1.8%, 2/113), ST120 (1.8%, 2/113), ST523 (1.8%, 2/113), ST557 (1.8%, 2/113), ST1411 (1.8%, 2/113), and ST1439 (1.8%, 2/113); the remaining STs contained 1 strain for each, except 34 novel STs (marked as NEW in Figure 1, currently not registered in the MLST database) as detected by MLST analysis (Figure 1). Also, 39 STs were analyzed for the molecular epidemiological relationships using goeBURST. The result showed that all 39 STs belonged to singletons with no CCs (Figure 2). In addition, of 16 ST190 strains, 4 isolates produced KPC-2, 1 produced NDM-1, 1 produced OXA-23, 1 produced IMP, and other produced ESBLs. All ST114 isolates produced NDM, including three (75%) strains with $bla_{\text{NDM-1}}$, and two produced NDM-5. All ST97 strains produced carbapenemase; three strains carried $bla_{\text{NDM-1}}$ and one bla_{IMP}.

Discussion

Carbapenem is widely used in the treatment and management of MDR Gram-negative bacterial infections in the clinical environment due to its broad-spectrum antibacterial activity.[19] However, some surveillance programs

Table 2 Minimum Inhibitory Concentrations (MICs) of 113 Carbapenem-Nonsusceptible ECC Isolates

Isolates	MIC (μg/mL)														
	ATM	CRO	CAZ	FEP	ETP	IPM	MEM	CIP	LVX	GEN	TOB	AMK	SXT	NIT	COL
Y509	≤1	≥64	≥64	≥64	16	2	4	1	1	≥16	≥16	8	≥320	/	0.25
Y541	64	≥32	≥128	32	2	0.5	≤0.125	0.06	0.06	4	2	4	≥320	64	8
Y542	32	≥32	≥128	4	2	0.5	0.25	0.25	0.06	2	0.5	2	20	64	0.125
Y569	32	≥32	≥128	4	1	≤0.125	≤0.125	0.06	0.06	2	0.5	2	≤5	32	0.125
Y572	32	≥32	≥128	1	2	0.5	0.5	0.06	0.06	2	2	8	10	32	0.25
Y573	≥128	≥32	≥128	8	2	0.5	0.25	≥8	≥16	64	16	≥512	≥320	128	0.25
CG35	0.5	≥32	≥128	16	2	8	4	0.25	0.5	8	8	4	≥320	32	0.125
CG38	1	≥32	≥128	32	64	64	64	0.5	1	16	8	4	≥320	32	0.25
CG64	32	≥32	≥128	32	8	4	4	≥8	≥16	4	16	4	≥320	32	0.25
CG80	64	≥32	≥128	8	1	1	0.25	1	1	4	4	1	≥320	64	0.125
CG85	0.5	4	16	0.06	4	2	0.25	0.5	0.06	4	4	8	≤5	64	0.125
CG98	64	16	8	8	128	8	16	≥8	≥16	32	4	4	≥320	≥1024	0.5
CG175	4	≥32	≥128	≥128	>128	64	64	1	1	8	8	4	≥320	128	>64
CG371	≥128	≥32	≥128	8	8	2	4	0.5	0.25	≥128	64	16	≥320	32	0.25
CG380	64	≥32	≥128	4	2	0.25	≤0.125	0.25	0.03	1	0.5	2	40	64	0.125
CG389	≥128	≥32	16	64	2	1	0.25	≥8	≥16	≥128	≥128	≥512	≥320	256	0.25
CG417	32	≥32	64	2	4	0.5	0.25	4	8	1	0.5	4	80	128	1
CG586	≤1	≥64	≥64	≥64	16	2	4	1	1	≥16	≥16	8	≥320	64	0.5
CG604	≥64	32	≥64	4	2	0.5	0.25	2	4	8	≥16	16	≥320	128	0.5
CG648	16	16	4	4	4	4	4	≤0.25	≤0.25	≤1	≤0.25	≤0.25	≤5	32	>64
CG652	32	≥32	≥128	2	2	0.25	≤0.125	0.25	0.5	2	1	4	≤5	64	0.25
CG662	8	≥32	≥128	8	2	4	1	0.25	0.25	64	4	4	≥320	16	0.5
CG666	32	≥64	≥64	4	2	0.25	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.25
CG698	≤1	≥64	≥64	8	2	1	0.5	≤0.25	1	8	4	≤2	≥320	64	>64
CG709	1	16	≥128	2	2	0.5	≤0.125	0.5	0.25	32	16	2	≥320	32	0.5
CG721	≥64	≥64	≥64	≥64	2	1	0.25	≥4	≥8	≤1	8	≤2	≤20	256	0.5
CG727	≥64	≥64	≥64	≤1	1	0.5	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	>64
CG737	≤1	4	2	≤1	4	0.5	≤0.125	≥4	≥8	≤1	8	≤2	≥320	64	>64
CG741	≤1	4	2	≤1	4	0.25	≤0.125	≥4	≥8	≤1	8	≤2	≥320	64	64
CG745	≥128	≥32	≥128	16	4	0.5	1	≥8	≥16	1	1	2	≥320	64	0.5
CG749	≤1	≥64	≥64	≥64	128	8	16	≥4	≥8	≥16	≥16	4	≥320	64	0.5
CG780	8	≥32	64	0.5	4	0.5	≤0.125	≥8	≥16	1	1	2	≥320	128	0.5

(*Continued*)

Table 2 (Continued).

Isolates	MIC (μg/mL)														
	ATM	CRO	CAZ	FEP	ETP	IPM	MEM	CIP	LVX	GEN	TOB	AMK	SXT	NIT	COL
CG804	≥128	≥32	≥128	64	32	4	4	≥8	≥16	2	1	4	≥320	32	0.5
CG815	≥64	≥64	16	8	16	16	4	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.5
CG817	≥64	32	16	2	8	4	1	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.5
CG701	2	≥64	2	32	2	2	0.25	≤0.25	1	≥16	8	≤2	≥320	32	>64
CG718	≤1	≤1	≤1	≤1	0.25	4	≤0.125	0.5	1	≤1	≤1	≤2	≤20	64	0.5
CG824	≥64	≥64	≥64	2	1	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.5
CG848	≥64	≥64	≥64	16	4	0.5	0,25	≥4	4	≤1	≤1	≤2	≤20	64	0.5
CG864	≥64	≥64	≥64	4	8	0.5	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	/	>64
CG871	≥64	≥64	≥64	2	2	0.5	0.125	≤0.25	0.5	≤1	≤1	≤2	≤20	/	0.5
CG884	≥64	≥64	≥64	≤1	2	0.5	≤0.125	≤0.25	1	≤1	≤1	≤2	≤20	/	32
CG901	64	≥32	≥128	64	16	2	2	≥8	8	64	≥128	≥512	≥320	256	0.5
CG911	≥64	≥64	16	16	16	8	4	0.5	1	≥16	≥16	16	≥320	64	0.5
CG914	≥64	≥64	16	16	32	4	4	0.5	1	≥16	≥16	16	≥320	64	0.5
CG916	≥64	≥64	≥64	≥64	128	16	16	0.5	1	≥16	≥16	≤2	≥320	64	0.5
CG934	≥64	≥64	≥64	≤1	2	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	≤16	>64
CG937	16	≥64	≥64	≤1	2	0.5	≤0.125	≥4	≥8	≥16	≥16	≥64	≥320	256	0.5
CG939	≥64	≥64	≥64	4	1	0.5	≤0.125	1	1	8	≥16	16	≥320	64	0.5
CG945	≥64	≥64	≥64	16	4	0.5	≤0.125	1	2	8	≥16	16	≥320	64	0.5
CG947	≥64	≥64	≥64	16	1	0.5	0.25	1	2	8	≥16	16	≥320	64	0.5
CG950	≥64	≥64	≥64	4	2	0.5	≤0.125	1	2	8	≥16	16	≥320	64	0.5
CG952	≥64	≥64	≥64	4	4	0.5	0.5	1	2	8	≥16	16	≥320	64	0.5
CG983	16	≥64	≥64	≥64	128	8	16	≥4	≥8	≥16	≥16	≥64	≤20	256	0.5
CG996	4	≥64	≥64	16	8	2	1	≤0.25	≤0.25	≥16	≥16	≤2	≤20	64	0.5
CG1005	≥64	≥64	≥64	≥64	>256	64	128	≥4	4	≥16	≥16	4	≥320	≥512	1
CG1015	≥64	≥64	≥64	2	1	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.25
CG1038	≥64	≥64	4	4	2	4	1	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	32
CG1041	≥64	≥64	≥64	2	1	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	33	0.5
CG1045	≥64	≥64	≥64	≥64	8	1	2	≥4	≥8	8	≥16	16	≥320	≤16	0.5
CG1050	≥64	≥64	≥64	≤1	2	0.5	≤0.125	1	1	≤1	≤1	≤2	≤20	32	16
CG1051	≥64	≥64	≥64	2	4	0.25	≤0.125	1	1	≤1	≤1	≤2	≤20	32	>64
CG1070	2	16	4	≤1	16	0.5	0.25	≥4	4	≤1	≤1	≤2	≤20	128	0.125
CG1075	≥64	≥64	≥64	≥64	32	2	2	≥4	≥8	≥16	8	≤2	≥320	64	0.25

(*Continued*)

Table 2 (Continued).

Isolates	MIC (μg/mL)														
	ATM	CRO	CAZ	FEP	ETP	IPM	MEM	CIP	LVX	GEN	TOB	AMK	SXT	NIT	COL
CG1079	≥64	≥64	≥64	≤1	4	0.5	0.5	≥4	4	4	2	8	≤20	128	0.125
CG1081	≥64	≥64	≥64	8	8	0.25	0.25	≥4	≥8	≥16	≥16	≤2	≥320	32	0.125
CG1090	≥64	≥64	≥64	≥64	16	1	4	≥4	≥8	≥16	8	≤2	≥320	64	0.125
CG1043	≤1	8	4	≤1	1	1	0.5	≥4	2	≥16	8	≤2	≥320	128	0.25
CG1159	4	32	4	2	4	0.25	≤0.125	1	1	8	4	≤2	≥320	64	0.5
CG1181	≥64	≥64	≥64	≤1	2	1	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.5
CG1208	≥64	≥64	≥64	4	4	0.5	0.25	2	4	4	4	≤2	≥320	128	0.5
CG1212	≥64	≥64	≥64	2	2	0.25	≤0.125	1	1	≤1	≤1	≤2	≤20	32	0.125
CG1231	≥64	≥64	≥64	2	4	0.25	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.25
CG1236	32	≥64	≥64	≤1	4	1	2	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.25
CG1244	≥64	≥64	≥64	≤1	2	0.25	0.5	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	0.125
CG1249	≥64	≥64	≥64	4	16	0.5	4	0.5	0.5	≤1	≤1	≤2	≤20	32	>64
CG1250	≥64	≥64	≥64	8	2	0.25	0.25	2	1	≤1	≤1	≤2	≥320	64	0.25
CG1252	16	16	16	≤1	1	0.5	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.5
CG1257	≤1	≥64	≥64	≥64	4	8	4	≤0.25	1	4	8	≤2	≥320	64	0.5
CG1280	≥64	≥64	≥64	2	1	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.125
CG1281	≥64	≥64	≥64	2	1	0.25	≤0.125	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.125
CG1330	≥64	≥64	≥64	≥64	16	4	4	≥4	≥8	4	4	≤2	≥320	64	0.125
CG1376	64	≥32	≥128	32	16	4	4	1	1	4	16	4	≥320	64	0.25
CG1381	≥64	≥64	≥64	≥64	16	16	8	0.5	1	4	8	≤2	≥320	32	0.25
CG1400	≥64	≥64	≥64	32	2	1	2	≥4	≥8	≥16	≥16	4	≥320	64	>64
CG1457	≥64	≥64	≥64	≥64	4	0.25	0.5	≤0.25	1	≤1	≤1	≤2	≤20	64	0.5
CG1461	≥64	≥64	≥64	16	4	2	1	≤0.25	1	≤1	≤1	≤2	≤20	128	0.5
CG1144	≥128	8	≥128	4	≤0.125	2	≤0.125	0.5	0.25	1	1	2	≤5	31	0.5
CG1479	≥64	≥64	≥64	8	128	16	8	≤0.25	≤0.25	≤1	≤1	≤2	≤20	32	>64
CG1498	16	≥64	≥64	≥64	4	16	1	2	1	4	8	≤2	≥320	32	0.5
CG1506	≥64	≥64	≥64	4	4	8	2	1	1	≥16	≥16	16	≤20	≤16	>64
CG1522	≥64	≥64	≥64	8	4	2	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	256	0.5
CG1532	≥64	≥64	≥64	4	2	1	0.25	0.5	1	8	8	≤2	≥320	64	0.5
CG1547	≥64	≥64	≥64	≥64	16	16	16	≥4	4	≤1	≤1	4	≥320	128	0.5
CG1563	≥128	≥32	≥128	1	2	1	0.25	1	1	2	1	2	≤5	16	0.25
CG1565	≥64	≥64	≥64	32	4	0.25	0.25	2	2	8	≥16	32	≥320	64	0.5

(*Continued*)

Table 2 (Continued).

Isolates	MIC (μg/mL)														
	ATM	CRO	CAZ	FEP	ETP	IPM	MEM	CIP	LVX	GEN	TOB	AMK	SXT	NIT	COL
CG1574	2	≥64	≥64	32	8	16	8	1	1	≤1	≤1	≤2	≤20	64	>64
CG1581	≥64	≥64	≥64	32	4	0.5	0.25	≥4	≥8	8	≥16	16	≥320	128	1
CG1589	≥128	≥32	≥128	4	2	0.5	0.25	≥8	≥16	≥128	≥128	≥512	≥320	128	0.5
CG1591	≥64	≥64	≥64	≥64	16	4	4	≥4	≥8	≤4	≥16	≤16	≥320	≤16	0.25
CG1593	32	≥64	≥64	≥16	2	8	2	0.5	1	≤1	8	≤2	≤20	≤16	0.5
CG1601	16	≥64	≥16	≤2	1	2	≤0.125	2	1	≥16	8	≤2	≥320	64	0.5
CG1606	≥64	≥64	≥64	≥64	8	2	1	≥4	≥8	≥16	≥16	≥64	≥320	256	1
CG1608	64	≥32	≥128	16	16	16	8	1	4	≥128	8	2	≤5	32	0.5
CG1640	≥64	≥64	16	8	>128	64	64	≥4	1	≤1	8	≤2	≤20	32	0.5
CG1728	≥64	≥64	≥64	4	4	0.25	0.25	≤0.25	≤0.25	≤1	≤1	≤2	≤20	64	0.5
CG1737	≤1	≥64	≥64	≥64	64	8	8	≥4	≥8	8	≥16	≤2	≥320	64	0.5
CG1746	≥128	≥32	64	32	4	0.5	≤0.125	≥8	≥16	≥128	32	4	≥320	64	0.5
CG1778	≤4	≥64	≥64	≥64	32	8	8	≥4	≥8	≤1	≤1	≤2	≥320	64	0.5
CG1779	≤1	≥64	≥64	≥64	16	8	8	≥4	≥8	≤1	≤1	≤2	≥320	/	0.5
CG1781	≤1	≥64	≥64	≥64	16	8	4	≥4	≥8	≤1	≤1	≤2	≥320	/	0.5
CG1813	8	≥64	≥64	32	16	2	4	1	2	8	8	≤2	≥320	/	0.25
CG1819	≤1	≥64	≥64	≥64	32	4	8	≤0.25	≤0.25	≤1	≤1	≤2	≤20	/	>64

Abbreviations: ECC, *Enterobacter cloacae* complex; ATM, aztreonam; CRO, ceftriaxone; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; ETP, ertapenem; CIP, ciprofloxacin; LVX, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; COL, colistin; NIT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole; FEP, cefepime; /, not available.

reported a significant increase in the resistance of carbapenems, making clinical treatment a great challenge.[20,21] In the present study, we collected 1314 ECC clinical isolates from the First Affiliated Hospital of Wenzhou Medical University from 2004 to 2018, and 113/1314 (8.6%) carbapenem-nonsusceptible ECC isolates were determined. The resistance of ECC strains to carbapenems showed a fluctuating upward trend from 2004 to 2018 (Table 1). In addition, the susceptibility of these 113 carbapenem-nonsusceptible ECC strains to commonly used antibacterial drugs showed that the 113 ECC strains showed a trend of multi-drug resistance as a whole. The high resistance to fluoroquinolones, cephalosporins, monobactams, and colistin, but low to amikacin, was found in our study, which corresponded to previous findings.[22,23] It is worth noting that colistin resistance rate was as high as 17.7%, which was related to inherent colistin resistance in the genogroups of the ECC.[23] Therefore, exploring the mechanism of these strains resistant to carbapenem is important so as to better prevent resistance. As far as I know, it is first time to report the analysis of molecular mechanisms and epidemiology of carbapenem-nonsusceptible ECC isolates with a longer period and large number of strains. The findings might provide a reference for the monitoring and control of carbapenem-nonsusceptible ECC isolates.

The production of carbapenemase is one of the carbapenem resistance mechanisms of ECC.[9] New Delhi metallo-β-lactamase (NDM), which is encoded by the gene bla_{NDM}, can lead to resistance to most β-lactam antibiotics, which was first found in New Delhi, India, in 2009.[24] bla_{NDM-1} is prevalent in the Indian region, and multiple bla_{NDM} alleles have been detected in hospitals in eastern China and Czech.[6,25,26] The present study also showed the

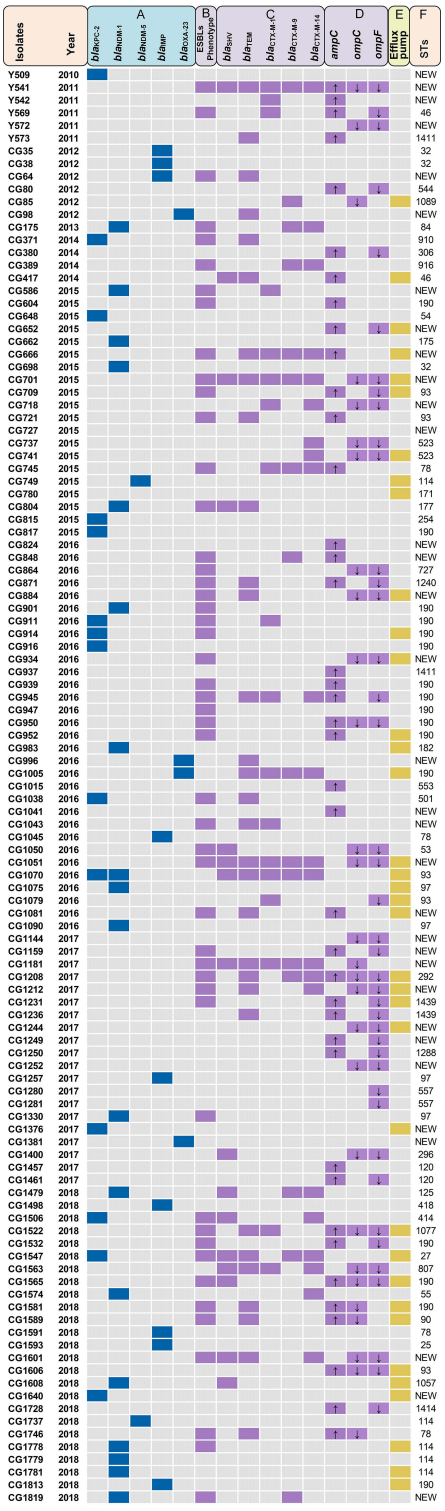

Figure 1 Antibiotic resistant mechanisms determined in the *Enterobacter cloacae* complex (ECC) isolates and multilocus sequence typing (MLST) in this study. (**A**) Carbapenem resistance genes; (**B**) extended-spectrum β-lactamases (ESBLs) phenotypic test for the phenotypic detection of ESBLs production; (**C**) β-lactam resistance genes; (**D**) expression of outer membrane porins genes and cephalosporinase gene *ampC*; (**E**) carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used to detect the activity of efflux pumps in carbapenem-nonsusceptible ECC isolates; (**F**) sequence typing (ST) of ECC. Blue, purple, and yellow squares represent positive. Gray squares represent negative. The purple square with an up arrow represents the overexpression of genes, and the purple square with a down arrow represents the low expression of genes.

emergence and spread of NDM-1, reflecting a high prevalence since 2012. The KPC enzyme was first discovered in ECC in 2005 and is extremely endemic worldwide now.[21,27,28] The dissemination of the ECC that produces KPC has been verified in China.[29,30] In addition, a previous study reported that 8.6% KPC-2-producing strains were detected in Chongqing.[31] However, our study showed that 12.4% (14/113) of the KPC-2-producing strains were found. VIM enzyme, which was reported in many regions, and OXA-48 enzyme, which was more common in Europe, were not found in the present study.[32,33] The study also indicated an increased number of carbapenemase-producing ECC isolates over the past few years, implying that the prevalence of carbapenem-nonsusceptible ECC was dependent on geographic regions and differences in drug use, even within a single country. The bla_{NDM} and bla_{KPC} were prevalent in carbapenemase-producing ECC in the hospital. Therefore, effective measures need to be taken to prevent the incidence.

In addition, ESBL genes were related to increased MIC to carbapenem, which were first identified in ECC in 1989.[34,35] Since then, the prevalence of ECC carrying ESBL has increased in hospital settings and in patients previously exposed to antibiotics.[36–38] Compared with non-ESBL-producing strains, ESBL-producing ECC showed a higher health risk associated with hospital-acquired infections.[39] In this study, 58.6% (66/113) ECC strains produced ESBLs; most of them had more than one ESBL gene. Moreover, the ESBLs gene bla_{TEM} was popular in this study, which was totally different from the predominant $bla_{\mathrm{CTX\text{-}M\text{-}15}}$ in Bulgaria.[40] We found that the carrying rates of ESBLs were higher in carbapenem-resistant ECC than in carbapenem-sensitive ECC, compared with other reports.[41]

The production of ESBLs or the overexpression of AmpC combined with disrupted membrane permeability (outer membrane protein decreased or loss) was another reason for carbapenem resistance.[3,9,42] In this study, 40 of 67 strains producing non-carbapenemase had the overexpression of AmpC or produced ESBLs combined with a decrease in the outer membrane proteins (Figure 1 and Table S2). Of the remaining 27 strains, 10 strains including 4 isolates with only decreased expression of *ompC* or/and *ompF*, 3 isolates with only overexpression of *ampC*, 2 isolates only producing ESBLs, and 1 isolate with ESBL and overexpression of *ampC* were intermediate to carbapenem, which correspond to a previous report that the

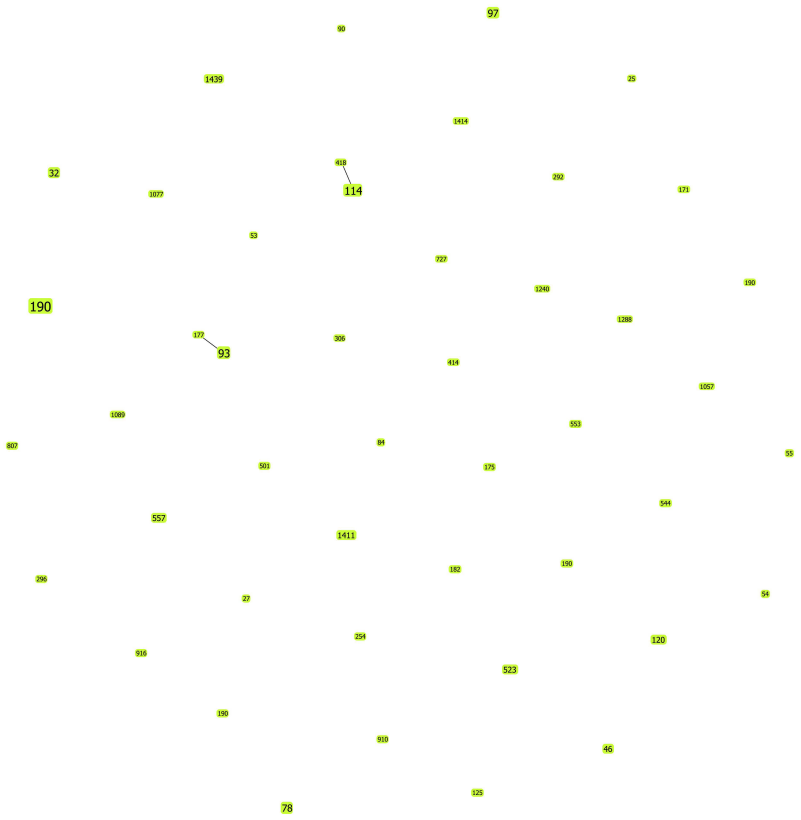

Figure 2 Performing goeBURST analysis on the molecular epidemiological characteristics of 79 ECC isolates. The population snapshot indicates the clonal assignment of the sequence typing (ST) presented in this study. Each green dot represents one ST, the numbers in the dot represent ST types, and the dot size represents their abundance in the ST set.

overexpression of *ampC* genes encoding cephalosporinase, leading to ECC strains resistant to first- and second-generation cephalosporins, and the disrupted membrane permeability (outer membrane protein decreased or loss) slightly increased MIC to carbapenem but did not lead to resistance.[35] However, 15 other ertapenem-resistant strains were also found in the aforementioned mechanism combinations. We suspected that these combinations might increase the MIC of these strains to carbapenem drugs and even reach the level of resistance (Table S2), which still requires further exploration.

Several studies reported the effect of the efflux pump on the carbapenem resistance of ertapenem-insensitive ECC strains.[9,42] The present study indicated that CCCP, an efflux pump inhibitor, decreased the MIC of 36 ECC strains to carbapenem, suggesting that efflux pumps played an important role in carbapenem resistance. However, the expression of the efflux pump acrB revealed no difference in ertapenem-resistant ECC strains, suggesting the presence of an additional unknown efflux pump influencing ertapenem resistance.[9]

The results of MLST analysis suggested that the distribution of STs of all carbapenem-nonsusceptible ECC isolates was diversified (Figure 2). ST190 was the most prevalent isolate in the hospital; a small-scale explosion in 2016 (Figures 1 and 2), different from previous studies, revealed that ST66, ST78, ST108, and ST114 were the most prevalent and widespread ECC STs.[35,43] The present study found that ST190 isolates producing carbapenemase and various other β-lactamase profiles with a higher risk might cause severe drug-resistant outbreaks in the hospital. ST78 and ST114 producing carbapenemase were major international clones, which were worth noting and reminded us of the spread of these strains.

In conclusion, this study summarized the resistance mechanisms and molecular epidemiology of carbapenem-nonsusceptible ECC strains in the hospital from 2004 to 2018. This was the first time that ECC nonsusceptible to carbapenem antibiotics was reported on a large scale in China. The increasing rates of resistance to antibiotics have further aggravated the threat to human health because of limited treatment options. ECC isolates that

do not produce carbapenemase are predominant in carbapenem-nonsusceptible ECC isolates. Carbapenem resistance is mediated by the overexpression of efflux pumps, or, more commonly, through the acquisition of constitutive overexpression of AmpC or ESBL combined with a decrease in the outer membrane proteins. The resistance of carbapenemase-producing ECC isolates is conferred through the acquisition of carbapenemase genes and the overexpression of efflux pumps. As carbapenem antibiotics are gradually applied as an effective treatment option, monitoring programs to prevent the emergence and further spread of antibiotic resistance are urgently needed.

Ethics Approval

The need for ethics approval and consent was deemed unnecessary in this study according to the ethics committee of the First Affiliated Hospital of Wenzhou Medical University.

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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 manuscript; gave final approval of the version to be published; have agreed on the journal to which the manuscript has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest.

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