Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular Mechanisms and Epidemiology of Carbapenem-Resistant *Escherichia coli* Isolated from Chinese Patients During 2002–2017

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Xuebin Tian[1,2]
Xiangkuo Zheng[2]
Yao Sun[1]
Renchi Fang[1]
Siqin Zhang[1]
Xiucai Zhang[1]
Jie Lin[1]
Jianming Cao[2]
Tieli Zhou[1]

[1]Department of Clinical Laboratory, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, People's Republic of China; [2]School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang Province, People's Republic of China

Background: The emergence and spread of carbapenem-resistant Escherichia coli (*E. coli*) pose a serious threat to human health worldwide. This study aimed to investigate the molecular mechanisms underlying carbapenem resistance and their prevalence among *E. coli* in China.

Methods: A collection of 5796 *E. coli* clinical isolates were collected from the First Affiliated Hospital of Wenzhou Medical University from 2002 to 2017. Sensitivity to antibiotics was determined using the agar dilution method. The detection of carbapenemases production and the prevalence of resistance-associated genes were investigated through modified carbapenem inactivation method (mCIM), PCR and sequencing. The mutations in outer membrane porins genes (*ompC* and *ompF*) were also analyzed by PCR and sequencing assays. The effect of efflux pump mechanism on carbapenem resistance was also tested. *E. coli* were typed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Results: A total of 58 strains (1.0%) of carbapenem-resistant *E. coli* were identified. The strains carrying $bla_{\text{KPC-2}}$ and bla_{NDM} accounted for 22.4% (13/58) and 51.7% (30/58), respectively. Among bla_{NDM}- positive strains, 27 bla_{NDM} genes were assigned to $bla_{\text{NDM-5}}$, while the remaining three strains were $bla_{\text{NDM-1}}$, whereas bla_{VIM}, bla_{IMP}, $bla_{\text{OXA-48}}$, and bla_{SHV} were not found. The CTX-M-type β-lactamase genes accounted for 96.6% (56/58). In addition, $bla_{\text{TEM-1}}$ genes were identified in 58.6% of tested strains. In carbapenem-resistant isolates, mutations in OmpC (the majority of mutated sites were D192G and Q104_F141del, accounting for 54.5%) and OmpF (large deletions S75_V127del, W83_D135del and Q88_D135del) were detected. Of note, the antibiotic resistance was not associated with overexpression of efflux pump. Moreover, MLST categorized the 58 carbapenem-resistant isolates into 19 different sequence types. PFGE analysis revealed that homology among the carbapenem-resistant isolates was low and sporadic.

Conclusion: The bla_{NDM} was the principal resistance mechanism of carbapenem-resistant *E. coli* in the hospital. $bla_{\text{NDM-5}}$ is becoming a new threat to public health and the alteration of outer membrane porins might help further increase the MIC of carbapenem.

Keywords: *Escherichia coli*, carbapenem-resistant, carbapenemase, outer membrane porin, epidemiology

Correspondence: Tieli Zhou
Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, People's Republic of China
Tel +86-0577-86699370
Email wyztli@163.com

Jianming Cao
School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, People's Republic of China
Tel +86-0577-88069595
Email wzcjming@163.com

Introduction

Escherichia coli is one of the most commonly isolated microorganisms in clinical specimens. Multidrug resistance in *E. coli* has become an upsetting issue observed in humans[1] and has been recognized as a contributor to the dissemination of antibiotic-resistance genes.[2] Controlling the dissemination of multidrug-resistant (MDR) strains is problematic due to very few new antibiotics available.[3,4] Because of increasing resistance

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to third-generation cephalosporins, fluoroquinolones and aminoglycosides, carbapenems have gradually become the last resort for life-threatening MDR *E. coli* infections because of their broad-spectrum antimicrobial agents.[5,6] Nevertheless, with an increasing consumption of carbapenems, the emergence of carbapenems resistant *E. coli* has become a serious public health concern worldwide.[7,8]

The mechanisms of carbapenem resistance are strongly associated with carbapenemase production (acquisition of carbapenemase genes), combination of porin loss with extended-spectrum β-lactamases (ESBLs) and the overexpression of efflux pumps.[9,10] Several studies have reported that acquired carbapenemase isolates might cause hospital outbreaks and become endemic in healthcare settings.[11,12] Globally predominant carbapenemases include KPC, NDM, VIM, IMP, and OXA, which are encoded by bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA} genes present in both the plasmid and the chromosome.[13,14] In addition, the carbapenemase genes could co-exist with ESBLs and other resistance genes on plasmids, which further limit the treatment options. Moreover, previous studies have reported that the outer membrane porins of *E. coli* are involved in the MDR phenotype.[15,16] Choi et al have constructed mutants of porins (*ompC* and *ompF* mutations) in *E. coli* and discovered that porins have a distinct role in antibiotic resistance and membrane integrity.[17] With the increase in the prevalence of carbapenem-resistant *E. coli* strains worldwide,[18,19] longitudinal epidemiological surveillance and mechanisms research on the carbapenem-resistant *E. coli* are of great clinical significance for the global control and prevention of the distribution and spread of resistance, as well as the guidance on antibacterial treatment. Nonetheless, there is still a lack of data on the long-term evolution of carbapenem-resistant *E. coli* in China. In the present study, we characterized the epidemiology prevalence and molecular mechanisms of 58 *E. coli* clinical isolates during large-scale surveillance for carbapenem resistance in the southeast of China.

Materials and Methods

Bacterial Isolates

A total of 5796 *E. coli* clinical isolates were collected from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between 2002 and 2017. Identification of all isolates was performed using a VITEK®2 system (bioMérieux, Marcy-l'Étoile, France). After collection, isolates were stored in 30% glycerol at –80°C. Relevant clinical data were collected from the medical records. We collected the information about isolation date, age, gender, sample, and ward.

Minimum Inhibitory Concentration Determination

MICs of 12 antimicrobial agents, including imipenem, meropenem, ertapenem, ampicillin, ceftriaxone, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, tobramycin, amikacin, and fosfomycin, were determined by the agar dilution method according to the guidelines recommended by the latest Clinical and Laboratory Standards Institute (CLSI).[20] Colistin MIC determination was performed with broth microdilution and interpreted by the recommendation of the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (http://www.eucast.org/). *E. coli* ATCC 25922 was used as the control strain for antimicrobial susceptibility testing.

Detection of Carbapenemases and Antibiotic Resistance Determinants

The modified carbapenem inactivation method (mCIM) was used to screen isolates for the production of carbapenemases, according to CLSI guidelines. The presence of resistant mechanisms, including carbapenem resistance genes (bla_{KPC-2}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}), ESBLs genes (bla_{SHV}, bla_{TEM}, $bla_{CTX-M-1}$, and $bla_{CTX-M-9}$), outer membrane porins genes (*ompC* and *ompF*), fosfomycin resistance genes (*fosA3* and *fosA*) and colistin resistance genes (*mcr-1* and *mcr-3*) were identified by polymerase chain reaction (PCR) and sequencing. Each isolate DNA was extracted from fresh bacterial colonies using a Biospin Bacterial Genomic DNA Extraction kit (Bioer Technology, Hangzhou, China). The primers used for amplification and sequencing were listed in Table S1. Positive amplification products were sent to Shanghai BGI Technology Co. (Shanghai, China) for sequencing. Nucleotide sequences were compared by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The online PROVEAN platform (http://provean.jcvi.org/seq_submit.php) was used to predict alterations in the biological function of the proteins.

Effect of Efflux Pump Mechanism on Carbapenem-Resistance in *E. coli*

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an energy uncoupler, has been identified as a compound to reverse MDR in *E. coli* over-expressing efflux pumps.[21] CCCP (Sigma, St Louis, MO) was used to measure the

activity of efflux pumps in carbapenem-resistant *E. coli* isolates. The change in the MICs of carbapenems was determined by the agar dilution method in the absence or presence of 10 μg/mL CCCP. A phenotype for positive efflux was defined as a ≧4-fold reduction of the carbapenem MIC in the presence of CCCP.[22]

Molecular Epidemiology Analysis

MLST analyses of the carbapenem-resistant isolates were carried out by amplifying eight housekeeping genes (*dinB, icdA, pabB, polB, putP, trpA, trpB*, and *uidA*). Sequence types were assigned by querying against the database available at the Institut Pasteur's *E. coli* MLST website (http://bigsdb.web.pasteur.fr/ecoli/ecoli.html).

To further identify potential clonal spread, PFGE was performed using a CHEF-Mapper XA PFGE system (Bio-Rad, Hercules, CA). Briefly, genomic DNA was extracted from all tested isolates, followed by *Xba* I restriction enzyme (Takara Bio, Inc., Kusatsu, Japan) digestion. Electrophoresis was then performed under the following conditions: temperature, 14°C; voltage, 6 V/cm; pulse angle 120°; and pulse duration, 2.16–54.17 s for 18.5 hrs. The universal standard strain *Salmonella enterica* serotype H9812 was used as a molecular marker.[23] Band patterns were analyzed and interpreted according to the criteria proposed by Tenover et al.[24]

Results

Bacterial Strains and Antimicrobial Susceptibility Testing

A total of 58 (1.0%) carbapenem-resistant *E. coli* isolates were identified with carbapenems (including imipenem, meropenem, and ertapenem), MICs ranging from 2 μg/mL to ≧16 μg/mL. Carbapenem-resistant *E. coli* isolates at our hospital were first detected in 2012; after that, the resistance rate has increased from 0.85% to 1.85% as was detected in 2017 (Table 1). Table 2 summarized the patient characteristics and species distribution. Overall, the carbapenem-resistant organisms were mainly from urine samples (31.0%, 18/58), followed by blood (27.6%, 16/58) and drainage (19.0%, 11/58). There were more isolates from males than females (62.1% vs 37.9%, respectively). Isolates were cultured from patients aged 19 to 91 years (average age 62.5 years). The majority of the isolates were from patients in the intensive care unit (ICU) (31.0%, 18/58), Hepatobiliary Surgery (10.3%, 6/58). The antimicrobial resistance profiles of the 58 carbapenem-resistant isolates were summarized in Table 3. According to the results of antimicrobial susceptibility testing, all 58 isolates showed higher resistance rates to cephalosporins, fluoroquinolones, and aminoglycosides. Thereinto, 55 (94.8%) isolates were resistant to fluoroquinolones, including levofloxacin and ciprofloxacin; 49 (84.5%) isolates were resistant to aminoglycosides, including gentamicin, tobramycin and amikacin. Furthermore, 58 (100%) isolates were resistant to ampicillin, while 18 (31.0%) isolates were resistant to fosfomycin and 2 (3.4%) to colistin.

Table 1 Carbapenems Susceptibility of E. coli Clinical Isolates

Time of Isolation	No. of Isolates	Resistant Strains (n)	S (%)	R (%)
2002	88	0	0	0
2003	163	0	0	0
2004	144	0	0	0
2005	134	0	0	0
2006	189	0	0	0
2007	300	0	0	0
2008	138	0	0	0
2009	145	0	0	0
2010	175	0	0	0
2011	185	0	0	0
2012	234	2	99.15	0.85
2013	211	0	0	0
2014	362	3	99.17	0.83
2015	747	6	99.20	0.80
2016	635	11	98.27	1.73
2017	1946	36	98.15	1.85

Abbreviations: No., number; S, sensitivity rate; R, resistance rate.

Prevalence of β-Lactamase Genes

Forty-three carbapenem-resistant *E. coli* isolates produced carbapenemases (Figure S1 and Table S2). The prevalence rates of bla_{KPC-2} and bla_{NDM} in carbapenem-resistant isolates were 22.4% and 51.7%, respectively (Figure 1), while bla_{IMP}, bla_{VIM} and bla_{OXA-48} were not detected. In addition, the number of isolates harbored bla_{NDM-1} or bla_{NDM-5} were 3 (5.2%) and 27 (46.6%), respectively. Moreover, the most prevalent CTX-M-type among analyzed strains was $bla_{CTX-M-1}$ (75.9%, 44/58), followed by $bla_{CTX-M-9}$ (65.5%, 38/58). In general, the CTX-M-type β-lactamase genes accounted for 96.6% (56/58). In addition to bla_{CTX-M} genes, bla_{TEM-1} genes were also identified in 58.6% of tested strains, bla_{SHV} was not detected.

Detection of Mutations in *ompC* and *ompF*

Mutations in *ompC* and *ompF* genes were detected in carbapenem-resistant isolates, including amino acid substitutions

Table 2 Patient's Clinical Data and Characteristics of Analyzed Strains

Strain	Isolation Date	Age	Gender	Sample	Ward
DC-38	03/03/2012	67	M	Drainage	Transplantation
DC-269	11/06/2012	75	M	Pus	Gastrointestinal Surgery
DC-1918	08/02/2014	77	F	Urine	Neurosurgery
DC-1960	21/02/2014	81	F	Pus	Endoscopy Center
DC-2003	06/03/2014	84	M	Blood	ICU
DC-3285	21/01/2015	77	M	Blood	ICU
DC-3737	05/05/2015	52	M	Wound	Operating room
DC-3835	24/05/2015	19	M	Blood	Hematology
DC-3938	19/06/2015	40	F	Blood	Hepatobiliary Surgery
DC-4069	18/07/2015	20	M	Blood	Hematology
DC-4385	06/10/2015	75	M	Wound	ICU
DC-4852	13/02/2016	87	M	Blood	Gastroenterology
DC-4967	19/03/2016	73	M	Urine	ICU
DC-5108	20/04/2016	68	F	Urine	ICU
DC-5113	21/04/2016	68	F	Blood	ICU
DC-5114	21/04/2016	73	M	Urine	ICU
DC-5127	22/04/2016	83	M	Pus	Anorectal Surgery
DC-5128	22/04/2016	67	F	Urine	Rehabilitation
DC-5147	25/04/2016	67	F	Blood	Emergency
DC-5178	02/05/2016	72	M	Drainage	Gastroenterology
DC-5183	05/05/2016	73	M	Urine	ICU
DC-5208	06/05/2016	72	M	Ascites	Gastroenterology
DC-6525	21/02/2017	48	M	Urine	Urology
DC-6581	02/03/2017	57	F	Urine	Neurosurgery
DC-6669	18/03/2017	76	M	Blood	ICU
DC-6729	01/04/2017	61	M	Drainage	Hepatobiliary Surgery
DC-6824	22/04/2017	51	F	Blood	Hematology
DC-6834	26/04/2017	76	M	Drainage	Hepatobiliary Surgery
DC-6856	29/04/2017	74	M	Urine	ICU
DC-6896	07/05/2017	74	M	Blood	ICU
DC-6899	07/05/2017	74	M	Urine	ICU
DC-6911	08/05/2017	73	F	Urine	Traumatology
DC-7114	10/06/2017	41	M	Wound	Gastrointestinal Surgery
DC-7143	13/06/2017	53	F	Drainage	Endoscopy Center
DC-7157	17/06/2017	48	M	Blood	Hematology
DC-7333	19/07/2017	47	M	Drainage	ICU
DC-7350	22/07/2017	33	F	Urine	Emergency
DC-7368	28/07/2017	58	F	Urine	Anorectal Surgery
DC-7523	24/08/2017	49	F	Drainage	Anorectal Surgery
DC-7603	08/09/2017	83	F	Blood	Hepatobiliary Surgery
DC-7658	20/09/2017	36	F	Urine	Urology
DC-7663	20/09/2017	41	M	Pus	Hepatobiliary Surgery
DC-7683	23/09/2017	80	M	Sputum	ICU
DC-7706	28/09/2017	58	M	Blood	Emergency
DC-7741	05/10/2017	59	M	Blood	ICU
DC-7781	13/10/2017	54	F	Drainage	Hepatobiliary Surgery
DC-7782	13/10/2017	67	F	Drainage	Transplantation
DC-7828	23/10/2017	91	M	Blood	Emergency
DC-7911	02/11/2017	46	M	Drainage	ICU
DC-7914	04/11/2017	46	M	Pus	Operating room
DC-7956	08/11/2017	46	M	Wound	ICU

(*Continued*)

Table 2 (Continued).

Strain	Isolation Date	Age	Gender	Sample	Ward
DC-7969	11/11/2017	83	F	Urine	Orthopaedic
DC-7980	14/11/2017	69	M	Drainage	Operating room
DC-7994	17/11/2017	71	M	Pus	Infectious diseases
DC-8085	04/12/2017	39	F	Urine	ICU
DC-8087	02/12/2017	42	M	Dialysate	Nephrology
DC-8111	06/12/2017	65	F	Urine	Urology
DC-8234	27/12/2017	87	F	Urine	Urology

Abbreviations: M, Male; F, Female.

and deletions. Deleterious mutations of OmpC and OmpF occurred in 22 and 21 isolates, respectively. Moreover, four carbapenem-resistant isolates had mutations in both OmpC and OmpF. The majority of mutation sites in *ompC* were D192G followed by Q104_F141del. Notably, several large deletions (S75_V127del, W83_D135del and Q88_D135del) of an amino acid sequence encoded by the *ompF* gene were also detected. Amino acid substitutions in *ompC* and *ompF* were considered deleterious by PROVEAN (Tables 4 and 5).

Phenotypic Detection of the Efflux Pump Overexpression

The effect of efflux pumps on the antibiotic resistance profiles of isolates was examined using the efflux pump inhibitor CCCP. When exposed to 10 μg/mL CCCP, none of the isolates showed a ≧4-fold decrease in the carbapenem MIC, suggesting that the antibiotic resistance was not associated with overexpression of efflux pump in this study.

Epidemiological Characterization

MLST analysis assigned the 58 carbapenem-resistant isolates into 19 different sequence types (STs) (Figure 2). ST8 was the predominant ST, accounting for 29.3% (17/58), followed by ST19 (12.1%, 7/58) and ST692 (12.1%, 7/58). Moreover, there were two novel STs (labelled as "New" in Figure 2; currently not registered in the MLST database). PFGE analysis revealed that homology among the resistant isolates was low and sporadic, suggesting a very low likelihood of clonal spread (Figure 2).

Discussion

Carbapenems are extensively applied in clinical settings for the therapeutic management of MDR Gram-negative bacterial infections due to their broad spectrum of antimicrobial activity.[25] Yet, several surveillance programs have reported a highly increasing carbapenem resistance, making clinical treatment more challenging.[26,27] In the current study, 58 of 5796 *E. coli* isolates exhibited an increasing carbapenem-resistant rate from 2002 to 2017. The relatively higher incidence revealed that the ongoing surveillance is urgently warranted in China.

From the clinical perspective, there have been reports of transmission of *E. coli* in the ICU,[28,29] and clinicians should be vigilant about the potential presence of this species. Our study also confirmed that carbapenem-resistant strains were most commonly isolated from patients aged >65 years who were treated in the ICU. The KPC-type enzyme was first reported in *Klebsiella pneumoniae* from the southern United States in 2001[30] and now endemic all over the world.[31,32] In China, dissemination of KPC-producing *Enterobacteriaceae spp*. has been confirmed in Shandong, Zhejiang, Taiwan, and other provinces.[33–35] KPC-2 was the most important in *K. pneumoniae*, whereas NDM-1 was the most important in *E. coli*. Notably, in previous studies in China, a few strains of *E. coli* with KPC-2 were detected.[36] However, in our study, 22.4% (13/58) of the strains were detected with KPC-2. This finding suggested that more attention should be paid to the spread of KPC-2 in this region. The IMP and VIM genes were reported in several regions, OXA-48 was more common in Europe but had not been found in our study.[37] New Delhi metallo-β-lactamase (NDM), which was first reported in Sweden in 2009 in a patient who developed an infection while travelling in India,[38] could confer resistance to most β-lactams. Over the recent years, a high prevalence of NDM-1 has been observed in China and India.[39,40] In addition, the rapid global spread of NDM-producing isolates via MDR plasmids has led many into thinking that common infections with such strains may soon be untreatable.[41] Selective pressure caused by

Table 3 Minimum Inhibitory Concentrations (MICs) of 58 Carbapenem-Resistant *E. coli* Isolates

Isolates	MIC (μg/mL)												
	AMP	CRO	CAZ	IPM	MEM	ETP	CIP	LVX	GEN	TOB	AMK	COL	FOS
DC-38	32	>64	16	0.125	0.25	2	0.5	1	>16	4	<2	0.25	2
DC-269	32	>64	16	0.5	2	16	>4	>8	>16	>16	<2	0.125	2
DC-1918	32	>64	16	0.125	0.25	4	>4	>8	>16	>16	<2	0.125	2
DC-1960	>32	>64	>64	0.125	2	2	>4	>8	>16	8	<2	0.125	2
DC-2003	>32	>64	>64	0.25	0.5	16	>4	>8	>16	>16	4	0.25	2
DC-3285	>32	>64	>64	8	4	16	>4	>8	>16	>16	>64	0.5	128
DC-3737	>32	>64	>64	8	16	16	>4	>8	>16	>16	16	16	1024
DC-3835	>32	>64	>64	4	16	16	>4	>8	>16	>16	<2	0.125	32
DC-3938	>32	>64	4	0.25	2	4	>4	>8	>16	8	<2	0.125	2
DC-4069	>32	>64	>64	4	16	16	>4	>8	>16	>16	>64	0.25	128
DC-4385	>32	>64	>64	0.25	2	8	>4	>8	>16	4	<2	0.125	1024
DC-4852	>32	>64	4	0.5	1	8	>4	>8	<1	<1	<2	0.125	8
DC-4967	>32	>64	16	2	2	8	>4	>8	>16	8	<2	0.25	2
DC-5108	>32	>64	>64	2	4	8	>4	>8	<1	<1	<2	0.125	2
DC-5113	>32	>64	>64	1	16	8	>4	>8	>16	8	<2	0.5	1
DC-5114	>32	>64	16	1	4	8	>4	>8	>16	8	<2	0.125	2
DC-5127	>32	>64	>64	0.125	0.125	2	>4	>8	>16	>16	32	0.25	1024
DC-5128	>32	>64	>64	4	16	16	>4	>8	>16	>16	32	0.5	1024
DC-5147	>32	>64	>64	1	1	8	>4	>8	>16	>16	4	0.25	2
DC-5178	>32	>64	>64	2	4	16	>4	>8	>16	>16	16	0.25	2
DC-5183	>32	>64	16	2	4	16	>4	>8	>16	8	<2	0.25	2
DC-5208	>32	>64	>64	1	4	16	>4	>8	>16	>16	16	0.25	2
DC-6525	>32	>64	16	0.06	0.125	2	>4	>8	>16	>16	<2	0.25	2
DC-6581	>32	>64	>64	0.5	8	16	>4	>8	>16	>16	8	0.125	1024
DC-6669	>32	>64	>64	0.5	0.5	8	>4	>8	>16	8	<2	0.25	2
DC-6729	>32	>64	>64	0.25	0.5	4	>4	>8	>16	>16	<2	0.25	1024
DC-6824	>32	>64	>64	1	2	8	>4	>8	>16	>16	16	0.25	2
DC-6834	>32	>64	>64	0.125	0.25	2	>4	>8	<1	<1	<2	0.25	2
DC-6856	>32	32	32	0.5	2	8	>4	4	4	2	<2	0.25	2
DC-6896	>32	>64	16	0.5	2	8	>4	>8	>16	8	<2	0.25	2
DC-6899	>32	>64	16	1	4	8	>4	>8	>16	8	<2	0.25	128
DC-6911	>32	>64	>64	1	1	8	>4	>8	>16	4	<2	0.25	2
DC-7114	>32	>64	>64	1	4	16	>4	>8	>16	8	<2	0.25	512
DC-7143	>32	>64	>64	4	4	8	>4	>8	<1	<1	<2	0.25	1024
DC-7157	>32	>64	>64	1	4	16	>4	>8	>16	>16	>64	0.25	512
DC-7333	>32	>64	>64	1	2	16	>4	>8	>16	>16	8	16	128
DC-7350	>32	>64	>64	1	8	16	>4	>8	>16	>16	>64	0.25	2
DC-7368	>32	>64	>64	2	2	16	>4	>8	4	8	<2	0.25	2
DC-7523	>32	>64	>64	2	4	16	>4	>8	>16	8	<2	0.5	512
DC-7603	>32	>64	>64	16	16	16	>4	>8	>16	8	<2	0.5	8
DC-7658	>32	>64	>64	2	8	16	>4	>8	>16	>16	>64	0.5	1024
DC-7663	>32	>64	>64	0.5	2	8	>4	>8	8	8	4	0.125	2
DC-7683	>32	>64	>64	1	2	8	>4	>8	>16	>16	>64	0.25	512
DC-7706	>32	>64	>64	2	4	16	>4	>8	>16	>16	>64	0.5	1024
DC-7741	>32	>64	>64	4	8	16	>4	>8	>16	8	<2	0.5	1
DC-7781	>32	>64	>64	1	4	16	>4	>8	>16	>16	8	0.25	1
DC-7782	>32	>64	>64	2	16	16	>4	>8	>16	>16	8	0.25	2
DC-7828	8	<1	<1	1	2	16	>4	>8	<1	<1	<2	0.25	2
DC-7911	>32	>64	>64	16	16	16	>4	>8	>16	>16	>64	0.25	1024

(*Continued*)

Table 3 (Continued).

Isolates	MIC (μg/mL)												
	AMP	CRO	CAZ	IPM	MEM	ETP	CIP	LVX	GEN	TOB	AMK	COL	FOS
DC-7914	>32	>64	>64	16	16	16	>4	>8	>16	>16	>64	0.25	1024
DC-7956	>32	>64	>64	16	16	16	>4	>8	>16	>16	>64	0.5	1024
DC-7969	>32	>64	>64	0.125	0.125	2	>4	>8	<1	<1	<2	0.125	2
DC-7980	>32	>64	>64	0.125	0.5	2	>4	>8	>16	8	<2	0.25	1024
DC-7994	>32	>64	4	0.5	1	2	>4	>8	>16	8	<2	0.25	4
DC-8085	>32	>64	>64	1	2	16	>4	>8	>16	8	<2	0.25	2
DC-8087	>32	>64	>64	1	4	16	0.5	1	>16	>16	4	0.25	2
DC-8111	>32	>64	>64	1	4	16	>4	>8	>16	>16	>64	0.5	512
DC-8234	>32	>64	>64	0.5	2	16	2	1	>16	8	<2	0.25	2

Abbreviations: AMP, ampicillin; CRO, ceftriaxone; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; ETP, ertapenem; CIP, ciprofloxacin; LVX, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; COL, colistin, FOS, fosfomycin.

increased use of antibiotics may drive the evolution of NDM-1, thus resulting in the emergence of its variants. In the current study, the emergence of NDM-5 reflected a new prevalence since 2017. M154L amino acid substitution in NDM-5 was the most common substitution in all NDMs variants,[42] responsible for increased carbapenemase activity. Moreover, NDM-5 has an extra V88L substitution; the emergence of V88L may contribute to lower catalytic activity on imipenem and meropenem.[43,44] Although NDM-5 made anti-infective treatment more difficult,[45] the lower hydrolytic activity of imipenem and meropenem implied these were still the first choice for MDR *E. coli* isolates. Our study indicated an increased number of carbapenemases-producing *E. coli* isolates over the last few years. It also revealed the high incidence of bla_{NDM} since it was first discovered at the hospital between 2015 and 2017. Interestingly, our results revealed that NDM-5 may even replace the NDM-1 in carbapenem-resistant *E. coli* isolates from 2017 in China. To date, several studies showed that bla_{NDM-5} was carried by IncX3 plasmids in China,[46,47] India,[48] Denmark[49] and Australia.[50] The fact that IncX-type plasmids have been shown to be conjugatable in most studies could explain the rapid spread of bla_{NDM-5}-carrying isolates. Therefore, it is imperative that feasible and effective measures are taken immediately.

ESBL-producing *E. coli* showed higher health risks related to hospital-acquired infections compared to non-ESBL-producing isolates.[51] CTX-M β-lactamases are the most widespread types of ESBLs, which have been identified in the mid-2000s in clinical *E. coli* isolates.[52] In this study, 96.6% of ESBL genes were classified as bla_{CTX-M}. Several reports have indicated that the transfer of CTX-M mobile plasmids could be frequently accompanied by the acquisition of fosfomycin resistance genes.[53,54] In our study, 17 carbapenem-resistant strains harboring CTX-M plasmids were positive for the *fosA3* gene. Colistin resistance represents another health concern. Two colistin-resistant *E. coli* strains detected in our study carried *mcr-1* gene. Moreover, co-harboring of bla_{NDM-1}, *fosA3*, and *mcr-1* were detected in DC-3737, like a reservoir, which posed serious concern on public health.

It has been reported that resistance to carbapenems could be mediated by non-specific outer membrane porins OmpC and OmpF in *E. coli*.[17] In the current study, the deleterious mutations were detected in 39 isolates, whereas OmpC and OmpF alteration occurred in 22 and 21 isolates, respectively. Mutation prediction showed that the amino acid substitutions in *ompC*, such as D192G might be the key factor driving resistance to carbapenems, while amino acid deletions could make an important impact in *ompF* mutations. The mutations in OmpF and OmpC were the important mechanisms contributing to the elevated MICs to carbapenems.

All of the isolates (100%, 58/58) were ertapenem non-susceptible; however, the abundance of imipenem-resistant strains was relatively smaller, promising the suitability of imipenem as the choice of treatment for infections caused by ertapenem-non-susceptible *E. coli* isolates. Furthermore, the alteration of outer membrane porins combined with carbapenemase production were found in 39 isolates, which further decreased the sensitivity of imipenem and meropenem. Otherwise, it is worth noting that the carbapenem resistance mechanism of DC-38 still remains unclear, and needs to be further researched in the future.

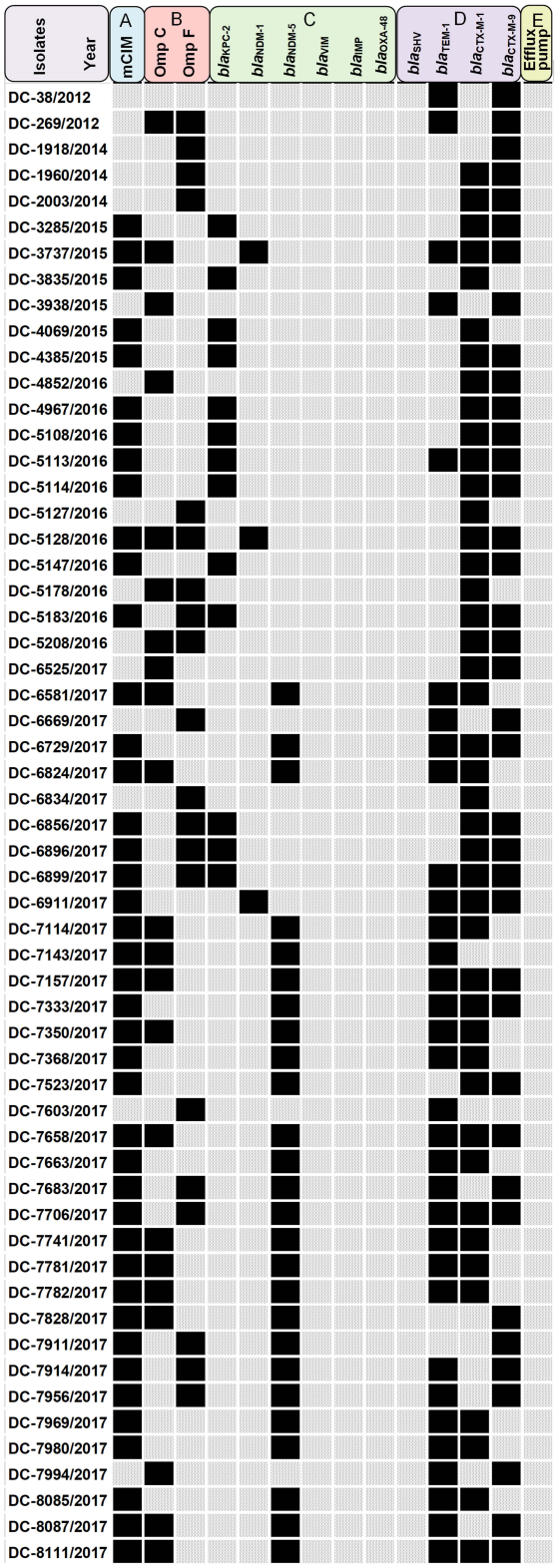

Figure 1 Antibiotic resistant mechanisms detected in the *E. coli* strains that were sequenced as part of this study. (**A**) Modified carbapenem inactivation method (mCIM) for phenotypic detection of carbapenemase production; (**B**) outer membrane porins genes; (**C**) carbapenem resistance genes; (**D**) β-lactam resistance genes; (**E**) carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to measure the activity of efflux pumps in carbapenem-resistant *E. coli* isolates. Black squares represent positive, gray squares represent negative.

Table 4 Mutations in Carbapenem-Resistant *E. coli* Isolates

Isolate	Amino Acid Substitution (s)	
	ompC	***ompF***
DC-38	–	–
DC-269	V359E	G206F
DC-1918	–	Y85N, N86del
DC-1960	–	P12_L14del
DC-2003	–	G206F
DC-3285	D350A	–
DC-3737	D350A, F367C	–
DC-3835	–	–
DC-3938	F367C	–
DC-4069	–	–-
DC-4385	–	–
DC-4852	Q104_F141del	–
DC-4967	–	–
DC-5108	–	–
DC-5113	–	–
DC-5114	–	–
DC-5127	–	S300_G309del
DC-5128	N47K	S300_G309del
DC-5147	–	N52D, A225E
DC-5178	Q104_F141del	L249_N252del
DC-5183	–	A13D
DC-5208	Q104_F141del	R257_L280del
DC-6525	D192G	N52D,
DC-6581	V15I, D126Y	–
DC-6669	–	N/d
DC-6729	–	–
DC-6824	D192G	–
DC-6834	–	A23_D34del
DC-6856	–	A23_D34del
DC-6896	–	A23_D34del
DC-6899	–	A23_D34del
DC-6911	–	–
DC-7114	D192G	–
DC-7143	D192G	–
DC-7157	D192G, Q104_F141del	–
DC-7333	–	–
DC-7350	D192G,	K28Q
DC-7368	G307_R308insVING	–
DC-7523	–	–
DC-7603	G307_R308insTIAG	Y128M
DC-7658	D192G,	–
DC-7663	V3A	–
DC-7683	–	W83_D135del
DC-7706	G307_R308insVING	S75_V127del
DC-7741	D192G	–
DC-7781	D192G	–
DC-7782	D192G	–
DC-7828	D192G	–
DC-7911	P177V, L296V	N27_K38del
DC-7914	P177V, L296V	W83_D135del
DC-7956	P177V, L296V	K241_T276del
DC-7969	–	–
DC-7980	–	–
DC-7994	D192G	N52D
DC-8085	–	–
DC-8087	D192G	–
DC-8111	D192G	–
DC-8234	–	N52D, Q88_D135del

Abbreviations: del, deletion; ins, insert; –, no mutation; N/d, failed to amplify.

Table 5 Analysis of Mutations in *ompC* and *ompF*

Gene	Mutations[a]	Comment[b]
ompC	V359E, F367C, Q104_F141del, N47K, D192G, D126Y	Deleterious
	V15I, D350A, G307_R308insVING, G307_R308insTIAG, P177V, L296V	Neutral
ompF	A23_D34del, G206F, S300_G309del, W83_D135del, Y85N, N86del, P12_L14del, L249_N252del, A13D, R257_L280del, Y128M, S75_V127del, N27_K38del, K241_T276del, Q88_D135del	Deleterious
	N52D, A225E, K28Q	Neutral

Notes: [a]del: deletion; ins: insert. [b]Predict by PROVEAN software and compared with sequences of ATCC 25922 in GenBank.

So far, few studies have reported the effect of efflux pump on carbapenems resistance in *Enterobacter spp*.[55–57] The current study showed that the efflux pump inhibitor CCCP was not able to restore the susceptibility of carbapenem-resistant *E. coli*, indicating that efflux pump was not involved in the carbapenem resistance in our study.

Our analysis showed that the majority of carbapenem-resistant clinical *E. coli* isolates showed different PFGE patterns, suggesting that they were genetically unrelated. The results of MLST demonstrated that these carbapenem-resistant isolates were polyclonal without a clonal dissemination. We speculated that carbapenem-resistant *E. coli* isolates might originate from different lineages and sources, instead of expansion of a single clonal lineage, which is in line with previous reports.[58] Among them, ST8 was the main clone type (29.3%, 17/58). Interestingly, 76.9% (10/13) KPC-2-producing *E. coli* isolates belonged

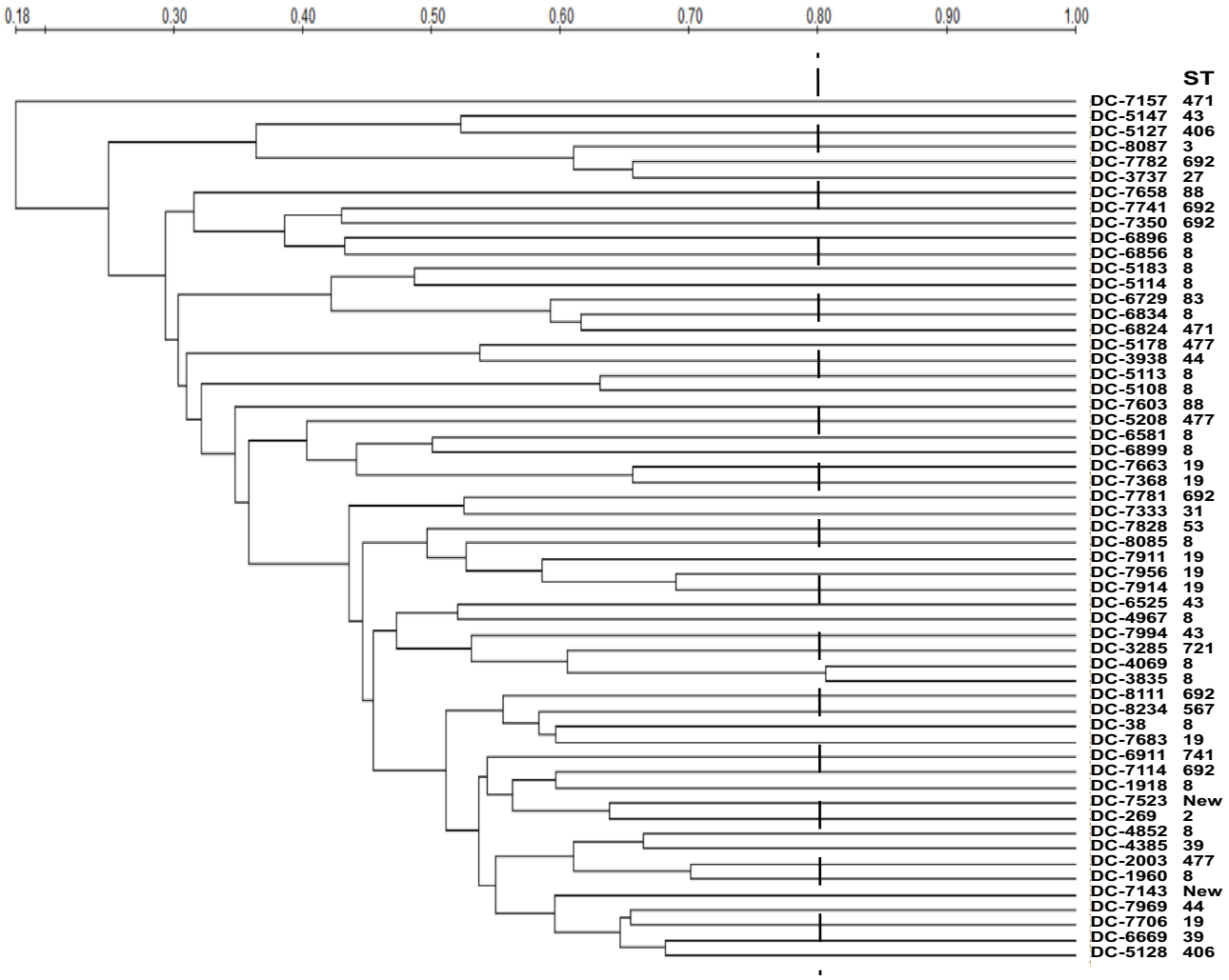

Figure 2 PFGE profiles of X*ba* I-digested chromosomal DNAs of carbapenem-resistant *E. coli* isolates. Relatedness was analyzed using QualityOne software (Bio-Rad Laboratories, USA). The phylogenetic tree was generated using UPGMA clustering. A genetic similarity index scale is indicated by the vertical line.

to ST8 in our study, indicated that a high prevalence of $bla_{\text{KPC-2}}$ was linked with ST8. We hypothesized that ST8 had a better ability to capture or accumulate bla_{KPC} compared with the other types. Furthermore, both STs ST19 and ST692 were present in association with the $bla_{\text{NDM-5}}$ gene, which was firstly reported to be linked with NDM-5-producing isolates.

In summary, we described the resistance mechanisms and the molecular epidemiology of carbapenem-resistant *E. coli* isolates at the First Affiliated Hospital of Wenzhou Medical University between 2002 and 2017. To best of our knowledge, this is the first report on the long duration and large scale of carbapenem-resistant *E. coli* isolates in China. Due to the limited treatment options, the rising resistance rate has further exacerbated the threat to public health. The prevalence of variant $bla_{\text{NDM-5}}$ represents a new threat. Moreover, ESBLs genes have shown to have a significant role in the carbapenem-resistant *E. coli* isolates, among which, CTX-M-type ESBLs were prevalent. As carbapenems are becoming ever more used as an effective therapeutic option, monitoring programs are urgently required to prevent the emergence and further spread of its resistance.

Ethical Statement

No samples were collected specifically for this research; only anonymized clinical residual samples collected during routine hospital procedures were used for this study.

Acknowledgment

We thank the Planned Science and Technology Project of Wenzhou (no. Y20170204) for providing financial funding.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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