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Original Article

Molecular and epidemiology characterization of carbapenem-resistant *Escherichia coli* in Hangzhou, China

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Abstract

Introduction: With the large-scale use of antibiotics, the detection rate and mortality of carbapenem resistant *Escherichia coli* (CR-EC) have gradually increased. This study investigated the molecular characteristics and prevalence of CR-EC in order to supplement the isolated data of CR-EC in Hangzhou, China.

Methodology: The minimal inhibitory concentration was determined by microbroth dilution method. The drug resistance genes were detected by polymerase chain reaction. The transferability of plasmid was verified by the conjugation test and genetic homology was detected by pulsed-field gel electrophoresis. The whole genome was sequenced (WGS) using the Illumina MiSeq technology.

Results: A total of 8 non-duplicated CR-EC isolates were collected, and all exhibited a multidrug-resistant phenotype. Two different *New Delhi metallo-β-lactamase* (*NDM*) variants, *blaNDM-5* and *blaNDM-13*, were found with detection rates of 62.5% and 12.5%, respectively. The success rate of conjugation was 100% (6/6). Homology analysis showed that there was no widespread cloning outbreak of CR-EC, and *blaNDM-5-ST410* was prevalent in the local area as a dominant group. WGS also indicated the rate of occurrence of resistance genes carrying resistance for more types of antibiotics, as well as exposed potential virulence risks.

Conclusions: This was a survey on the prevalence and molecular characteristics of CR-EC in Hangzhou. *blaNDM*-like production combined with extended spectrum beta-lactamase (ESBLs) and/or AmpC was the main resistance mechanism of CR-EC in this area. The dominant *blaNDM-5-ST410* requires enhanced attention. The horizontal transformation of plasmids, complex drug resistance, and potential virulence risks also need close attention.

Key words: carbapenem; resistant; *NDM*; conjugation; transfer.

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Introduction

Bacterial resistance has become a major challenge to the global public health [1,2]. Carbapenem resistant *Escherichia coli* (CR-EC) is one of the main pathogens of carbapenem-resistant Enterobacteriaceae (CRE) causing various clinical infections, such as respiratory, urinary, and bloodstream infections [3]. The 2019 report by the Centers for Disease Control and Prevention (CDC) [4] noted that the infection situation caused by CR-EC had not improved compared to the previous years. Similarly, the China Antimicrobial Surveillance Network (CHINET) reported that untreatable and hard-to-treat infections from CR-EC were on the rise among patients in medical facilities [5]. The occurrence of CR-EC has been documented in multiple regions across the globe; however, the prevalence and spread of CR-EC exhibited notable regional variation [6]. Previous studies have shown that ST167, ST405, ST410, ST361, and ST648 are widespread in Europe [7]. ST131, ST167, and ST410 have been mostly reported in China [5], however, the relevant data on CR-EC in Hangzhou area is still incomplete.

This study aimed to characterize CR-EC strains from a tertiary hospital in Hangzhou, China, focusing on their resistance genes, plasmid profiles, and epidemiological patterns. This information would help to understand the resistance patterns and transmission

situation of CR-EC in this area. The research outcome and experimental data provide theoretical support for further understanding of the pathogen, guiding clinical rational use of antibiotics, and effective prevention and control of infection.

Methodology

Collection and identification of bacterial isolates

The VITEK 2 compact automated system (bioMerieux, Marcy l'Etoile, France) and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS; bioMérieux, Marcy l'Etoile, France) were applied for isolate identification. The Clinical and Laboratory Standards Institute (CLSI) guidelines [8] define CR-EC as resistant to any carbapenem drug (including imipenem (IMP), meropenem (MEM), and ertapenem (ETP)). All *Escherichia coli* that conformed to this standard were included in this study. All collected CR-EC were reviewed by the E-test method and stored in glycerol at – 80 °C for further investigation.

Antimicrobial susceptibility testing

Minimum inhibitory concentration (MIC) is defined as the lowest compound concentration (μg/mL) required to stop bacterial growth. The MIC values of the 23 antibiotics in 12 categories, including carbapenem, were determined by the microbroth dilution method with a Gram-negative stain drug-susceptible-plate (Shandong Xinke Biology, Shandong, China). The experiment was carried out according to the manufacturer's instructions. ATCC 25922, ATCC 700603, and BAA-1705 were used as quality control strains; sterile broth was the negative control; and antibiotic-free bacterial fluid was the positive control. Each sample was tested 3 times in parallel to exclude operating errors. All interpretation results were valid under normal quality control conditions. The results were interpreted based on the CLSI criteria; however, the tigecycline (TIG), colistin (CT), and polymyxin (PB) results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint recommendations.

Detection of resistance genes

DNA was crudely extracted by the boiling method. All resistance genes were detected by polymerase chain reaction (PCR), including carbapenemase genes (bla_{NDM}, bla_{IMP-4}, bla_{IMP-8}, bla_{KPC}, bla_{VIM-1}, bla_{VIM-2}, and bla_{OXA-48}), extended spectrum beta-lactamase (ESBL) genes (bla_{CTX-M}), and other genes (*mcr-1* and *mcr-9*). The primer sequences are detailed in Supplementary Table 1. Enzyme-free water was used as a blank control. The positive amplification products were sent to Shanghai Shenggong Biotechnology Co., Ltd. (Shanghai, China) for Sanger sequencing, and the sequencing results were compared using the Basic Local Alignment Search Tool (BLAST), available at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Conjugation experiment

The conjugation experiment was carried out using a membrane bonding experiment as previously described [9,10]. The donor (CR-EC) and the recipient (EC600) strains were mixed on Luria-Bertani agar at a ratio of 1:3, and the mixtures were incubated for 24 hours at 35 °C. Transconjugants were selected in LB broth supplemented with rifampicin (600 μg/mL) and meropenem (1 μg/mL). DNA was extracted from the transconjugants grown on the double-antibody plate, and bla_{NDM} was amplified by PCR. The transconjugant, donor and recipient were tested by pulsed-field gel electrophoresis (PFGE). When the transconjugant verified by PCR was consistent with the recipient pattern, but not consistent with the donor pattern, it was regarded as successful conjugation. The carrier status of other drug resistance genes and the MIC values of the transconjugants were determined by PCR and antimicrobial susceptibility testing.

Pulsed-field gel electrophoresis (PFGE)

PFGE was used to further determine the genetic relatedness based on the previously published protocol [11]. CR-ECs were digested with *XbaI* for 4 hours at 37 °C. The digested fragments were separated on a 1% Seakem Gold agarose gel (BiORAD, Hercules, USA) for 18 hours at 14 °C using the Bio-Rad CHEF MAPPER® XA System [12]. The band patterns were analyzed using BioNumerics Software version 7.0 [13]. The patterns were identified as previously described, and clusters were defined as DNA patterns sharing ≥ 80% similarity [14].

Whole-genome sequencing (WGS) and data analysis

Genomic DNA was prepared using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany); and NanoDrop2000 (Thermo Scientific™, Waltham, MA, USA) was used to measure DNA concentration and quality, with pure water without nuclease as a blank control. WGS was performed with the MiSeq platform (Illumina Inc., San Diego, CA, USA) with PE-150 type. FastQC v0.11.9 was used for quality control [15], and genome assembly was performed using Shovill v1.1.0 [16]. The sequences were scanned with the MLST

Table 1. Clinical features of CR-ECs.

Isolate number	Gender	Age (years)	Ward	Specimen	Clinical diagnosis	Clinical outcome	Antibiotic exposure
CR-EC3089	F	79	ICU	Urine	Pulmonary infection	Death	Yes
CR-EC3276	M	56	Hepatology Department	Blood	Ascites	Improvement	Yes
CR-EC4082	M	69	Surgical Department	Bile	Cerebrovascular disease	Discharge	Yes
CR-EC5129	F	73	Surgical Department	Urine	Kidney stone	Improvement	No
CR-EC6301	F	45	Infectious Diseases Department	Blood	AIDS	Discharge	Yes
CR-EC6332	F	55	Endocrinology Department	Urine	Diabetes	Discharge	Yes
CR-EC6428	F	50	Oncology Department	Blood	Liver cirrhosis	Death	Yes
CR-EC6512	F	70	Respiratory Department	Sputum	Pulmonary infection	Discharge	Yes

CR-EC: carbapenem resistant *Escherichia coli*; F: female: M: male: ICU: intensive care unit; AIDS: acquired immunodeficiency syndrome.

v2.19.0 tool [17] to obtain the multilocus sequence typing (MLST) subtypes. Plasmid Finder 2.1 was used for plasmid replicant typing [18], and ResFinder [19,20] and VirulenceFinder [21] were used to identify resistance genes and virulence genes, and predict resistance phenotypes and the virulence situation.

Nucleotide sequence accession numbers

The complete sequences of all the strains were submitted to GenBank. The accession numbers of the eight CR-ECs are: SAMN41314880, SAMN41314881, SAMN41314882, SAMN41314883, SAMN41314884, SAMN41314885, SAMN41314886, and SAMN41314887 respectively.

Results

Characteristics of CR-EC

A total of 8 non-duplicated CR-EC isolates were investigated. Multiple isolates from the same patient at the same site were not included. The detection rate of blood and urine was the same (37.5%). The samples were collected from 7 different clinical departments, and the surgical department accounted for a relatively higher proportion of samples (25%). The average age of infected individuals was 62 years, 75% of them were over 50 years old, and most of them were male (75%). 87.5% of patients had a history of antibiotic exposure. The details are summarized in Table 1.

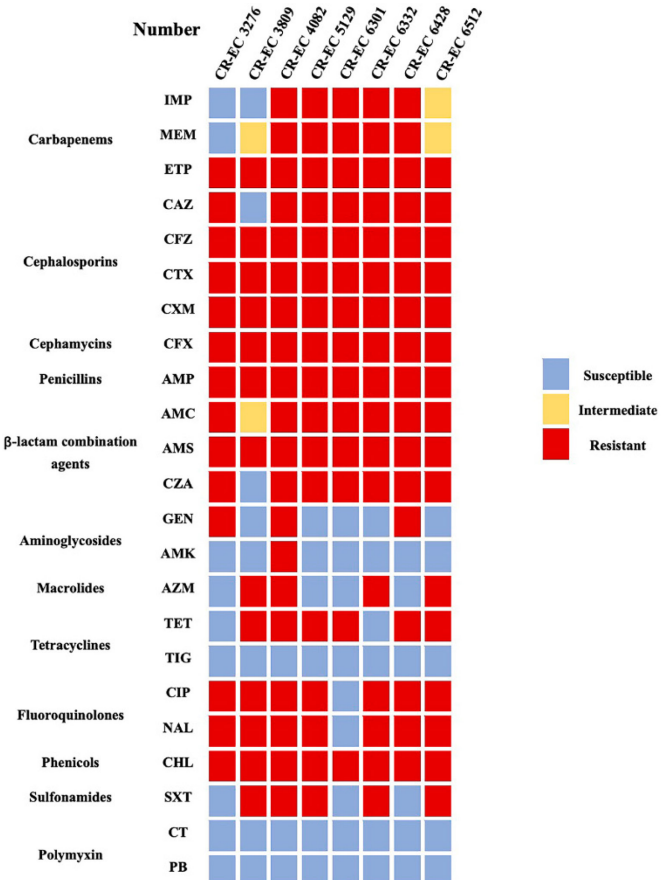
Antimicrobial susceptibility testing

All experimental strains were classified as multidrug-resistant (MDR) bacteria (Figure 1) [2]. The resistance rate of CR-EC to ertapenem was 100%, while the resistance rate to imipenem and meropenem was relatively reduced (62.5% for both). Nevertheless, the resistance rate of CR-EC can reach almost 100% for traditional antibiotics such as cephalosporins, penicillins, and β-lactam combination agents. In addition, CR-EC also showed high resistance to quinolones (87.5%) and chloramphenicol (100%).

Detection of resistance genes

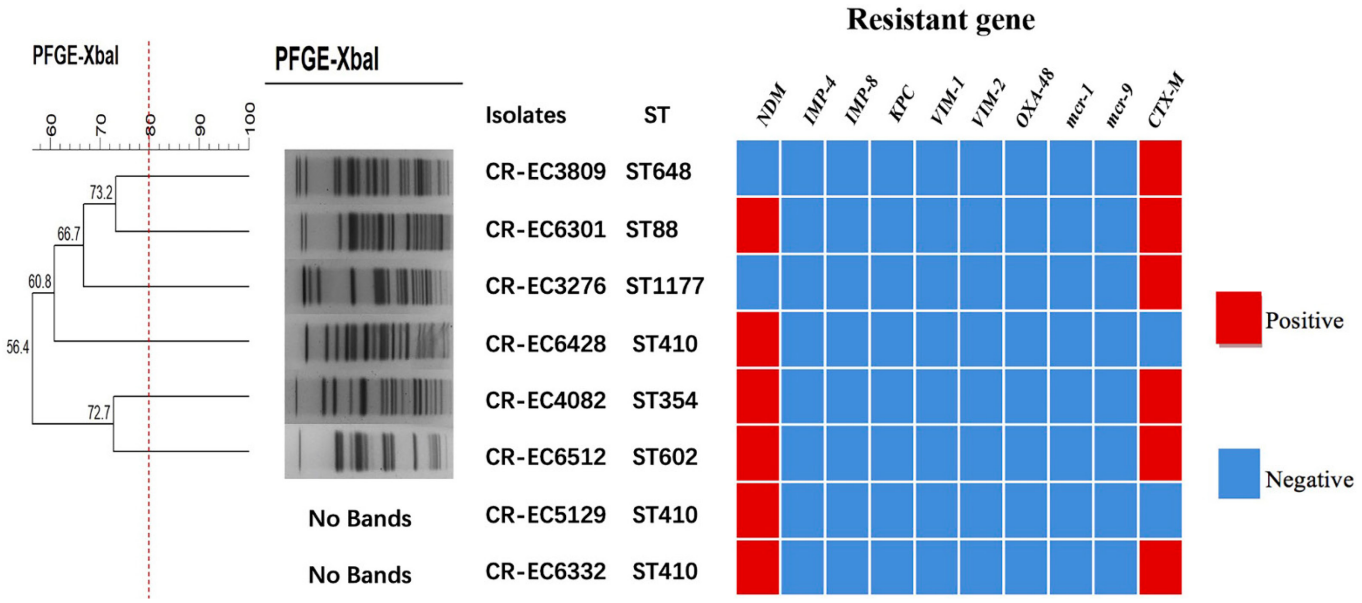
The carbapenem resistance gene *blaNDM* had the highest detection rate at 75%. After confirmation of the sequences by Sanger sequencing, the proportions of *blaNDM-1*, *blaNDM-5*, and *blaNDM-13* were 0% (0/8), 62.5% (5/8), and 12.5% (1/8), respectively. Other common class A, B, and D carbapenem genes were not

Figure 1. Antimicrobial susceptibility testing of CR-EC.



IMP: imipenem; MEM: meropenem; ETP: ertapenem; AMP: ampicillin; CAZ: ceftazidime; CFZ: cefazolin; CTX: cefotaxime; CXM: cefuroxime; CFX: cefoxitin; AMC: amoxicillin-clavulanate; AMS: ampicillin-sulbacatam; CZA: ceftazidime-avibactam; GEN: gentamicin; AMK: amikacin; AZM: azithromycin; TET: tetracycline; TIG: tigecycline; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; SXT: trimethoprim-sulfamethoxazole; CT: colistin; PB: polymyxin B.

Figure 2. Homology and presence of resistance genes.



ST: sequencing type; PFGE-*Xba1*: pulsed-field gel electrophoresis-Xba1 enzyme. PFGE-*Xba1* value represents the corresponding percentage. Clusters were defined as DNA patterns sharing ≥ 80% similarity.

Table 2. Susceptibility results of various antibiotics for donors (CR-EC) and transconjugants (μg/mL).

Isolate number	CR-EC isolate						*E. coli* transconjugant strain					
	CR-EC4082	CR-EC5129	CR-EC6301	CR-EC6332	CR-EC6428	CR-EC6512	*J4082*	*J5129*	*J6301*	*J6332*	*J6428*	*J6512*
Resistance genes	bla_{NDM-5} $bla_{CTX-M-55}$ $bla_{CTX-M-14}$	bla_{NDM-5}	bla_{NDM-13} $bla_{CTX-M-14}$	bla_{NDM-5} $bla_{CTX-M-55}$	bla_{NDM-5}	bla_{NDM-5} $bla_{CTX-M-27}$	bla_{NDM-5}	bla_{NDM-5}	bla_{NDM-13}	bla_{NDM-5}	bla_{NDM-5}	bla_{NDM-5}
IMP	4	4	8	4	> 8	2	4	4	8	4	8	2
MEM	> 4	> 4	> 4	> 4	4	2	4	> 4	4	> 4	4	2
ETP	> 8	> 8	> 8	> 8	8	4	> 8	> 8	> 8	> 8	4	4
CAZ	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
CFZ	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
CTX	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16
CXM	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
CFX	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
AMP	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
AMC	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32
AMS	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	> 64/32	32/16	32/16	> 64/32	> 64/32	> 64/32
CZA	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4	> 8/4
GEN	> 32	≤ 1	≤ 1	≤ 1	16	≤ 1	16	≤ 1	≤ 1	≤ 1	16	≤ 1
AMK	> 64	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	> 64	≤ 2	≤ 2	2	≤ 2	≤ 2
AZM	> 64	8	8	64	16	64	> 64	8	4	32	8	32
TET	> 32	> 32	> 32	4	> 32	> 32	> 32	> 32	16	2	> 32	> 32
TIG	0.5	≤ 0.25	≤ 0.25	1	1	1	≤ 0.25	≤ 0.25	≤ 0.25	0.5	0.5	1
CIP	> 32	> 32	0.25	> 32	> 32	8	> 32	> 32	≤ 0.25	> 32	> 32	4
NAL	> 64	> 64	8	> 64	> 64	> 64	> 64	32	4	> 64	> 64	> 64
CHL	> 64	> 64	> 64	> 64	8	> 64	> 64	> 64	> 64	> 64	8	> 64
SXT	> 8/152	> 8/152	0.5/9.5	> 8/152	≤ 0.25/4.75	> 8/152	> 8/152	> 8/152	≤ 0.5/9.5	> 8/152	≤ 0.25/4.75	> 8/152
CT	0.25	≤ 0.25	0.25	0.25	0.25	0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.25	≤ 0.25
PB	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12

IMP: imipenem; MEM: meropenem; ETP: ertapenem; AMP: ampicillin; CAZ: ceftazidime; CFZ: cefazolin; CTX: cefotaxime; CXM: cefuroxime; CFX: cefoxitin; AMC: amoxicillin-clavulanate; AMS: ampicillin-sulbacatam; CZA: ceftazidimeavibactam; GEN: gentamicin; AMK: amikacin; AZM: azithromycin; TET: tetracycline; TIG: tigecycline; CIP: ciprofloxacin; NAL: nalidixic acid; CHL: chloramphenicol; SXT: trimethoprim-sulfamethoxazole; CT: colistin; PB: polymyxin B; CR-EC: carbapenem resistant *Escherichia coli*.

detected. (Figure 2). In addition, the ESBL class gene *blaCTX-M* also had an extremely high detection rate (75%). Among the *blaCTX-M genes*, *blaCTX-M-14* and *blaCTX-M-55* had the highest frequency, both accounting for 37.5%. *blaCTX-M-27* was also identified at a lower frequency (12.5%). The genes related to colistin resistance (*mcr-1* and *mcr-9*) were not detected.

Results of plasmid conjugation tests

CR-EC3809 and CR-EC3276 did not carry *blaNDM*. Therefore, a total of 6 strains were tested for conjugation, and the conjugation success rate was 100% (6/6). All *blaNDM*-like were transferred from donor to recipient, while the *blaCTX-M*-like was unable to transfer. The availability of *blaNDM*-like greatly increased the sensitivity of carbapenems, penicillins, and β-lactam combination agents; and the MIC values were the same or slightly lower than that of donor. The specific results are detailed in Table 2.

Homology comparison

PFGE analysis was conducted using the unweighted pair group method and the arithmetic mean (UPGMA) using a dice coefficient to judge the strain's affinity, which had a similar PFGE pattern that exceeded similarity coefficient between bacteria (SAB) 0.8; thus indicating that the isolates shared a clonal relationship. All of the samples had similarity of < 80%, indicating that they were polyclonal and had different origins (Figure 2). It is worth noting that no matter how the experimental process of PFGE, CR-EC5129, and CR-EC6332 were adjusted, clear and complete bands could not be obtained. Such results are likely related to the characteristics of the bacterial isolate itself, such as rapid degradation of nucleic acid, biochemical characteristics of strains, etc.

ST410 was the most common type (37.5%, 3/8); while, the detection rates of ST648, ST88, ST1177, ST354, and ST602 were all 12.5%.

Sequencing

The GC content of all strains was in the range of 50–55%, after quality control and assembly of all sequencing data. In addition to *blaNDM*-like and *blaCTX-M*-like discovered by PCR; ESBLs *OXA*-like, *SHV*-like, *TEM*-like, and AmpC:CMY-like were also discovered. Besides the common aminoglycoside, quinolone, and fosfomycin resistance genes, 7 different antibiotic-resistance genes were also identified in CR-EC. A total of 9 plasmid replicators were detected. Among them, 3 strains carried *IncFIB* replicators, 3 strains carried *IncX3* replicators, 2 strains carried *IncHI2* replicators. The other types of replicons were: *IncFIA*, *IncFII*, *IncN*, *IncI1-1*, *IncX4* and *IncFIC* (Table 3).

The distribution of virulence factors in CR-EC was also not optimistic. A total of 6 functional proteins were predicted with "identity = 100%" as the standard. According to the phylogenetic analysis method established by Clermont *et al*. [22], *Escherichia coli* can be divided into 4 evolutionary groups (A, B1, B2, D) [23]. Among them, 2 strains belong to Group D, and 6 strains belong to Group A. In addition, genes related to adhesion and invasion (*fimH*), outer membrane (*traT*), and iron metabolism (*iutA* and *sitA*) were identified with detection rates of 100%, 87.5%, 75% and 100%, respectively. The types of toxic factors contained in CR-EC are also relatively complex, as detailed in Table 4.

Table 3. The various drug resistance genes and plasmid types for CR-EC.

Number	Beta-lactam	Aminoglycoside	Fluoroquinolone	Tetracycline	others	Plasmid replicators
CR-EC3276	$bla_{CTX-M-55}$	/	/	/	/	Col156
CR-EC3809	$bla_{CTX-M-14}$	*aph(6)-Id aph(3")-Ib aadA5*	/	*tet(A) tet(B)*	*mph(A) floR sul2 sul1*	IncFIA IncFIB IncFII Col(BS512)
CR-EC4082	bla_{OXA-1} bla_{NDM-5} $bla_{CTX-M-55}$ $bla_{CTX-M-14}$	*armA aac(6')-Ib-cr aac(3)-IId aph(6)-Id aph(3")-Ib aadA22 aph(3')-Ia*	*qnrS1*	*tet(A)*	*fosA3 msr(E) mph(E) mph(A) lnu(F) catB3 floR ARR-3 ARR-2 sul1 sul2 sul3 dfrA14*	IncX3 IncHI2
CR-EC5129	bla_{CMY-2} $bla_{TEM-214}$ $bla_{TEM-206}$ $bla_{TEM-141}$ bla_{TEM-1B} bla_{NDM-5}	*aph(6)-Id aph(3")-Ib*	*OqxB OqxA*	*tet(A)*	*fosA3 sul2 bleO*	IncX3 IncFIB Col(BS512)
CR-EC6301	$bla_{CTX-M-14}$ bla_{NDM-13} bla_{OXA-10}	*aadA1*	*qnrS1*	*tet(A)*	*floR cmlA1 ARR-2 dfrA14*	IncHI2
CR-EC6332	bla_{CMY-2} $bla_{CTX-M-55}$ $bla_{TEM-214}$ $bla_{TEM-206}$ $bla_{TEM-141}$ bla_{TEM-1B} bla_{NDM-5}	/	/	/	*fosA3*	IncX3 IncN Col(BS512)
CR-EC6428	bla_{NDM-5} bla_{OXA-1}	*aph(4)-Ia aac(3)-IV aac(6')-Ib-cr*	/	*tet(A)*	*catB3 ARR-3 sul1*	IncI1-I IncX4
CR-EC6512	bla_{NDM-5} $bla_{CTX-M-27}$	*aadA2 aph(6)-Id aph(3")-Ib*	/	*tet(B)*	*fosA7 fosA3 mph(A) catA1 sul1 sul2 dfrA12*	IncFIB IncFIC p0111

CR-EC: carbapenem resistant *Escherichia coli*.

Table 4. The various virulence related genes of CR-EC.

Classification of virulence genes	Number							
	CR-EC3276	**CR-EC3809**	**CR-EC4082**	**CR-EC5129**	**CR-EC6301**	**CR-EC6332**	**CR-EC6428**	**CR-EC6512**
Related to adhesion and invasion	*afaD* (afimbrial adhesion)	/	*usp* (Tia Invasion determinant)	/	/	/	/	/
	fimH (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)	*fimH* (Type 1 fimbriae)
	/	*tia* (Tia Invasion determinant)	*lpfA* (Long polar fimbriae)	/	*lpfA* (Long polar fimbriae)	/	*lpfA* (Long polar fimbriae)	*lpfA* (Long polar fimbriae)
	/	*yfcV* (Fimbrial protein)	*yfcV* (Fimbrial protein)	/	/	/	/	/
Related to outer membrane	*chuA* (Outer membrane hemin receptor)	*OmpT* (Outer membrane protease (protein protease 7))	*chuA* (Outer membrane hemin receptor)	/	*OmpT* (Outer membrane protease (protein protease 7))	/	*OmpT* (Outer membrane protease (protein protease 7))	/
	/	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)	*traT* (Outer membrane protein complement resistance)
Related to capsule	*kpsMII_K5* (Polysialic acid transport protein; Group 2 capsule)	*kpsMII_K5* (Polysialic acid transport protein; Group 2 capsule)	/	/	/	/	/	/
	/	*KpsE* (Capsule polysaccharide export inner-membrane protein)	*KpsE* (Capsule polysaccharide export inner-membrane protein)	/	/	/	/	/
Related to iron metabolism	*fyuA* (Siderophore receptor)	*fyuA* (Siderophore receptor)	/	*iroN* (Enterobactin siderophore receptor protein)	*fyuA* (Siderophore receptor)	/	*fyuA* (Siderophore receptor) *iroN* (Enterobactin siderophore receptor protein)	*fyuA* (Siderophore receptor)
	iutA (Ferric aerobactin receptor)	*iutA* (Ferric aerobactin receptor)	*iutA* (Ferric aerobactin receptor)	/	*iutA* (Ferric aerobactin receptor)	*iutA* (Ferric aerobactin receptor)	*iutA* (Ferric aerobactin receptor)	/
	sitA (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)	*sitA* (Iron transport protein)
Related to toxin	*hra* (Heat-resistant agglutinin)	/	*astA* (Heat-stable enterotoxin EAST-1)	/	*astA* (Heat-stable enterotoxin EAST-1)	/	/	/
	/	/	/	*hlyF* (Hemolysin F)	*hlyF* (Hemolysin F)	/	*hlyF* (Hemolysin F)	*hlyF* (Hemolysin F)
	/	/	*cea* (Colicin E1)	/	*cea* (Colicin E1)	*cma* (Colicin M activity)	/	/
related to transfer	/	/	/	*traJ* (Positive regulator of conjugal transfer operon)	/	/	/	/

() represents the corresponding protein function. CR-EC: carbapenem resistant *Escherichia coli*.

Discussion

Carbapenems are antibiotics used to treat MDR bacterial infections as a last resort. CR-EC are one of the most common and important carbapenem-resistant Gram-negative bacteria. In this study, CR-ECs were mainly isolated from urine and blood samples, which is consistent with other reports [24]. This indicates that CR-EC was widely distributed in clinical practice in the hospital and may cause multiple site infections, which should be strengthened in attention and management.

Based on the resistance mechanism, CR-EC can be divided into carbapenemase-producing *E. coli* (CPE) and non-carbapenemase-producing *E. coli* (non-CPE) [25]. Compared with non-CPE, CPE has a 3–6 times higher infection mortality rate and stronger transmission ability [26,27]. The main reason for the widespread resistance in *E. coli* is that they carry *NDM* [28,29]. This study revealed three *NDM* variants, and their main differences are shown in Table 5. It is worth noting that the difference in amino acid sequences between *NDM-5* and *NDM-1* results in a stronger hydrolysis activity of *NDM-5* towards carbapenems [30]. NDM-13 has 4 additional mutation sites compared with *NDM-1*. *NDM-13* only has two mutation sites compared with NDM-5, which did not increase the hydrolytic activity of *NDM-13* towards carbapenems, cephalosporins, and penicillins; but increased its affinity for cefotaxime and affected the catalytic activity of the enzyme towards cefotaxime [31]. This indicates that among the three variants, *NDM-5* still has strong hydrolytic ability, while CR-EC carrying *NDM-5* is more likely to exhibit resistance phenotype. *blaNDM-5* exhibits stronger resistance to a wider range of antibiotics. Therefore, in an environment with the selective pressure of multiple antibiotics, bacteria carrying *NDM-5* have a greater survival advantage, making them more likely to become prevalent. In addition, research indicates that *NDM-5* is often located on mobile genetic elements characterized by low adaptive cost and high transfer efficiency. These elements can be transferred efficiently between different bacteria, which greatly promotes the prevalence and spread of *NDM-5* [32]. In contrast, *NDM-13* may lack such highly efficient transmission elements, or the elements such as plasmids where it resides have relatively weak transfer and dissemination capabilities, thus limiting its spread within the bacterial population. In this study, the detection rate of *blaNDM*-like was 75% and this confirmed the main resistance mechanism of local CR-EC. *blaNDM-5* was the dominant variant, which is consistent with a multicenter study in China [5].

NDM-producing strains that combine with ESBLs and/or AmpC can be a problem. 25% of the CR-ECs were triple carriers of NDM-ESBLs-AmpC, and 50% were double carriers of NDM-ESBL. Among the ESBLs, the CTX-M type was predominant, mainly including the *CTX-M-14* and *CTX-M-55* types, which is consistent with the domestic trend. CMY plasmid-mediated-AmpC have also been reported in *E. coli* [33]. Complex enzyme production is reflected in the MDR phenotype and increases the difficulty of clinical anti-infection treatment. Previous studies have reported that CR-EC has high resistance to non-beta-lactam antimicrobials such as aminoglycosides, fluoroquinolones, and tetracycline; in addition to carbapenems [34]. In this study, CR-EC also showed high resistance rates to cephamycins (100%), chloramphenicol (100%), fluoroquinolones (87.5%), tetracyclines (75%) and sulfonamides (62.5%). Both the resistance rates and genetic profiles are consistent with previous studies [35]. Fortunately, regardless of genotype or phenotype, CR-ECs still exhibit high sensitivity to polymyxin antibiotics. The use of polymyxin alone and/or in combination can be considered as one of the last resort measures for treating CR-EC infection [36], but further research is still needed on the medication strategy.

The success of conjugation experiments demonstrated that *NDM* was located on transferable plasmids and that resistance could be transmitted between species through horizontal transfer [37]. All *CTX-M* cannot be transferred along with *NDM*, indicating that *CTX-M* is not located in the same mobile plasmid as *NDM*, and may also be located on chromosomes. This conclusion is consistent with previous research [10]. The mild decline of the resistance phenotype of the transconjugant also confirmed that the formation of CR-EC was the result of a combination of mechanisms [38,39], such as

Table 5. Differences between New Delhi metallo-β-lactamase (NDM) variants.

NDM variants*	Discovery area	Reporting period	Strain or genus	GeneBank sequence number	Non-synonymous substitution	Amino acid substitution
NDM-1	India	2009	*Klebsiella pneumoniae*	FN396876	-	-
NDM-5	Britain	2011	*Escherichia coli*	JN104597	G262T, A460C	V88L, M154L
NDM-13	Nepal	2014	*Escherichia coli*	LC012596	G283A, A460C	D96N, M154L

*Nucleotide and amino acid positions (in comparison to *NDM-1*) of nonsynonymous substitutions were listed. Amino acid abbreviations follow the standard single letter code.

deletion and mutation of porin *OmpC*, decreased expression and affinity of penicillin-binding-protein, and increased efflux pump activity; which could not be transferred with mobile elements. Therefore, it is important to pay close attention to the trend and migration of CPE with transfer function. Although, the conjugation success rate of *NDM-5* (5/5, 100%) was the same as that of *NDM-13* (1/1,100 %), it was speculated that the differences between *NDM* variants might be related to the different types of plasmids. As far as *blaNDM* is concerned, it can be located on plasmids with multiple replicators in host bacteria [40–42], and *blaNDM-1* is mostly located on *IncN* and *IncFIIA* [43]. *blaNDM-5* is mostly located on *IncX3*, *IncII*, *IncFIB*, *IncFIC*, and *IncX4* [40,44]; meanwhile, *blaNDM-13* is mostly located on *IncX3* [45]. These conclusions are also highly consistent with the results of this study. It is speculated that different replicators may have different transfer efficiencies. Although *blaNDM* has similar conserved sequences (△ISAba125-blaNDM-like-bleMBL-trpF-dsbC) [29,46], complex regulation of upstream and downstream genes may also cause differences between variants. It is impossible to obtain more detailed visualizations due to the limitations of the MiSeq-sequencing technology; therefore, further research will be conducted in the future.

Disturbingly, in addition to the terrifying drug resistance, it was found that CR-EC is quietly spreading in a form that carries potentially virulent risk pathogens. The presence of virulence factors helps bacteria survive, adapt, and evade the host environment and defense mechanisms, and is more likely to cause widespread infections and outbreaks [47]. The expression of special structural proteins such as capsular, flagella, and fimbriae enhances the invasion ability of CR-EC, which often adheres to a variety of infection sites and mediates the adhesion of in vitro interventional substances, such as ventilators and catheters [48]. Iron is an important component of host life activity, which can be used to resist pathogenic bacteria [49]. At the same time, iron is also an essential nutrient element for bacterial growth and reproduction, and bacteria can directly obtain iron from the host, thereby enhancing virulence or obtaining stronger survival ability in harsh environments. Therefore, bacterial virulence is closely related to iron [50]. Although the virulence of group A and group D in this study was lower than that of group B2 [51], the various enterotoxins carried by them indicate the potential virulence risk of CR-EC in the local region. Therefore, the dynamic changes of CR-EC should be monitored closely to prevent and control the production and transmission of highly virulent strains.

ST410, as a dominant clone group, is been widely spread in many cities in China. Zhuo *et al.* identified a CR-EC ST410 clone which caused two separate outbreaks in a children's hospital [52]. Fortunately, the PFGE and MLST results showed that there was no widespread cloning outbreak of CR-EC in this study. Consistent with other reports [5], *blaNDM-5-ST410* was prevalent in the local area as a dominant group, suggesting the need to strengthen monitoring of NDM-5-producing-ST410 *E. coli*. No other high-risk clone groups (ST167, ST617, ST405) in addition to ST410 were detected [24,53]; and other diverse typing was mostly reported in southern China, which also indicated that CR-EC was not mainly transmitted by cloning, but through the transmission of conjugable plasmids. Although there was no widespread transmission of CR-EC in the hospital, the presence of *blaNDM-5-ST410* high-risk clones still emphasizes the necessity of hospital infection control measures such as hand hygiene, contact isolation, and active monitoring.

There are several limitations to this study. Firstly, due to the impact of COVID-19 during the sample collection period of this study, there were insufficient patients with relevant cases, making it impossible to collect more strains. Additionally, this study was a single-center study, and the results cannot be applied to other regions. The relatively small sample size did not allow for in-depth statistical analysis and testing. Limited laboratory resources constrained the ability to explore the structure of mobile elements and transcriptomic analysis.

Conclusions

This study was a survey on the prevalence and molecular characteristics of CR-EC in Hangzhou. The study highlights the prevalence of bla_{NDM-5} and $bla_{CTX-M-55}$ in CR-EC isolates from Hangzhou, China, with *IncX3* and *IncFIB* plasmids playing a major role in resistance dissemination. In view of the widespread prevalence and outbreak of the ST410 clonal population in China, as well as the prevalence of CR-EC carrying *NDM* gene in the hospital, strengthening surveillance and infection control measures is necessary to combat the spread of these MDR strains. Such measures may include staff training on hand hygiene and disinfection, regular disinfection of environmental surfaces, and active screening and monitoring of high-risk groups.

This study provides theoretical data for guiding clinical rational treatment of CR-EC, and provides evidence that suggests that the combination of polymyxin and other drugs should be considered to

avoid further deterioration of antibiotic resistance. The transferability of plasmids exacerbates the spread of drug resistance. The high detection rate of virulence factors such as fimbriae and iron suggests potential virulence risk. Future studies will focus on more in-depth analysis of CR-EC samples, including sequencing of plasmids, to fully understand the molecular characteristics of CR-EC.

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Authors' Contributions

JL conceived and designed the study. LZ and KH wrote this paper and contributed equally to this work. LZ, KH, ZZ, MX, SL and JW performed the experiments. HY, JY, WZ and QX analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

No conflict of interest is declared.

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Annex – Supplementary Items

Supplementary Table 1. Primers used in this study.

Target gene	Primer name	Primer sequence
NDM	*NDM-F*	TCGCCCCATATTTTTGCTACAG
	NDM-R	CGATCCTTCCAACTCGTCGC
IMP-4	*IMP-4-F*	ACCGCAGCAGAGTCTTTGCC
	IMP-4-R	ACAACCAGTTTTGCCTTACC
IMP-8	*IMP-8-F*	GTTTTATGTGTATGCTTCC
	IMP-8-R	AGCCTGTTCCCATGTAC
KPC	*KPC-F*	TGTCACTGTATCGCCGTC
	KPC-R	CTCAGTGCTCTACAGAAAACC
VIM-1	*VIM-1-F*	AGTGGTGAGTATCCGACAG
	VIM-1-R	ATGAAAGTGCGTGGAGAC
VIM-2	*VIM-2-F*	ATGTTCAAACTTTTGAGTAAG
	VIM-2-R	CTACTCAACGACTGAGCG
OXA-48	*OXA-48-F*	TTGGTGGCATCGATTATCGG
	OXA-48-R	GAGCACTTCTTTTGTGATGGC
CTX-M	*CTX-M*	GGTTTAAAAAATCACTGCGTC
	CTX-M	TTGGTGACGATTTTAGCCGC
mcr-1	*mcr-1-F*	AGTCCGTTTGTTCTTGTGGC
	mcr -1-R	AGATCCTTGGTCTCGGCTTG
mcr-9	*mcr -9-F*	CTTTCCATAACAGCGAGACAC
	mcr -9-R	GTATCCTTCCTGCCATCCTC