

Carbapenemase- and Colistin Resistant *Escherichia coli* Strains from Children in China: High Genetic Diversity and First Report of *bla*_{NDM-5}, *bla*_{CTX-M-65}, *bla*_{OXA-10}, *bla*_{TEM-1}, and *mcr-1.1* Genes Co-Occurrence in *E. coli* ST156

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Background: The emergence of carbapenem and colistin co-resistant *Escherichia coli* poses a huge challenge to infection control. The purpose of this study was to clarify the mechanism of the carbapenems and colistin co-resistance in *E. coli* strains.

Methods: Antimicrobial susceptibility test was carried out by agar dilution methods and colistin resistance was confirmed by broth microdilution methods. Whole genome sequencing was carried out, and resistance genes, sequence types and virulence genes of carbapenems and colistin co-resistance *E. coli* isolates were analyzed.

Results: The results showed that among the 176 carbapenem-resistant *Enterobacteriaceae* strains, 5 multidrug resistant *E. coli* strains exhibiting coresistance to carbapenem and colistin. The main mechanism of 5 *E. coli* strains in this study was generating carbapenem. Four *E. coli* strains were *mcr*-positive, while one *mcr*-negative strain had a new *MgrB* mutation. The *bla*_{NDM-5}, *bla*_{CTX-M-65}, *bla*_{OXA-10}, *bla*_{TEM-1} and *mcr-1.1* genes were simultaneously detected in *E. coli* 20IR1127 strain belonging to ST156 lineage. Other antimicrobial resistance genes encoding aminoglycosides-, sulfonamide-, chloramphenicol-, tetracyclines- and macrolides resistance were also detected.

Conclusion: The main mechanisms of carbapenem and colistin resistance were encoded by *bla*_{NDM} and *mcr1.1*, meanwhile *mgrB* mutations also contribute to colistin resistance. To our knowledge, this study is the first to report of *E. coli* ST156 strain in which the *bla*_{NDM-5}, *bla*_{CTX-M-65}, *bla*_{OXA-10}, *bla*_{TEM-1} and *mcr1.1* genes coexist. In addition, there is also an *E. coli* ST457 strain, which carries *bla*_{TEM-1}, *bla*_{NDM-9}, *bla*_{CTX-M-199} and is positive for *mcr1.1* gene.

Keywords: *Escherichia coli*, carbapenem, colistin, co-resistance, WGS

Introduction

Multidrug resistance in *Escherichia coli* has become a concerning issue that is increasingly observed in human but also in veterinary medicine around the world.¹ *E. coli* is essentially sensitive to almost all clinically related antibiotics, but this bacterium has a great ability to accumulate antibiotic resistance genes, mainly through horizontal gene transfer.² The most serious resistance mechanisms in *E. coli* is related to the acquisition of genes coding for carbapenemases, extended-spectrum β -lactamases, plasmid-mediated quinolone resistance (PMQR) genes, and *mcr* genes (conferring resistance to polymyxins).³ The existence of plasmid-mediated antimicrobial resistance genes significantly increases the spread of carbapenem resistance, while further limiting the choice of effective antimicrobials. The spread and emergence of carbapenem-resistance caused by carbapenemase producing *Enterobacteriaceae* (CPE) and the deficiency of development of new antibiotics have led to the re-use of colistin in the

treatment of patients with CPE-related infections.⁴ Colistin is a cationic amphiphilic lipopeptide antibacterial agent, which is an important antibiotic for human treatment of multiple antibiotic-resistant (MDR) Gram-negative rod-shaped bacteria,^{5,6} as been referred to as “a last-resort antimicrobial”. Colistin has good activity against all kinds of gram-negative rod-shaped bacteria in vitro, including MDR *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*.⁷ According to literature reports, the *mcr*-mediated polymyxin resistance rate in clinical cases is about 1%, and the polymyxin resistance rate caused by bacterial chromosome mutation is 0.67%-1.6%.⁸ Although the antibiotic resistance rate of colistin in population is not high, with the global spread of colistin resistance gene *mcr*, the clinical application of colistin is under serious threat.

Used colistin in the treatment of carbapenem-resistant bacteria in human patients has given rise to an increase in colistin resistance, which is due to the change in lipid A of lipopolysaccharides caused by chromosome point mutation.⁹ The emergence and increase of carbapenem and colistin co-resistant to *E. coli* is an urgent problem to be studied.¹⁰

The purpose of this study is to illustrate the mechanism of the carbapenems and colistin co-resistance in *E. coli* strains. We collected co-resistant *E. coli* clinical isolates from a children's hospital, and performed this study through methods such as whole genome sequencing (WGS), antimicrobial susceptibility testing, to provide further understanding for the resistance development of *E. coli* strains. Finally, we aimed to clarify the mechanism of the carbapenems and colistin co-resistance in carbapenem and colistin co-resistance *E. coli* strains obtained from children crowd using WGS.

Materials and Methods

Bacterial Isolates and Colistin Resistance Screening

A 176 clinical Carbapenem-Resistant *Enterobacteriaceae* (CRE) isolates were recovered from the Children Hospital, Zhejiang University School of Medicine, from 2015 to 2020. All isolates were identified by MALDI-TOF MS using a Bruker Biotyper mass spectrometer (Bruker Daltonics, Germany). The study has been approved by the Ethics Committee of the Children's Hospital (2021-IRB-031), Zhejiang University School of Medicine.

The colistin-resistant isolates (MIC > 2) were selected to confirm the resistance phenotype by colistin microdilution in cation-adjusted Mueller-Hinton broth according to standardized methods (EUCAST, <http://www.eucast.org/>). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* 27,853 was used as quality control. The results showed that five strains of *E. coli* were co-resistant to carbapenem and colistin.

Antimicrobial Susceptibility Testing

The MIC of six other antibiotics including cefepime (FEP), ceftazidime (CAZ), levofloxacin (LVX), amikacin (AMK), imipenem (IPM) and piperacillin/tazobactam (TZP) in all carbapenem and colistin-resistant *E. coli* strains were further detected by broth dilution method. All antibiotic sensitivity results were interpreted according to the interpretation criteria of the Institute of Clinical and Laboratory Standards (CLSI).¹¹

Whole Genome Sequencing and Genome Assembly

E. coli strains co-resistant to carbapenem and colistin were identified by Whole Genome Sequencing (WGS), and the genetic characteristics and ST type were determined. Particularly, we determined multi-locus sequence type (MLST), virulence- and antibiotic resistance genes carriage. DNA was purified from carbapenem and colistin co-resistance isolates using QIAGEN-QiaAmp DNA Mini kit (QIAGEN, Hilden, Germany). DNA was quantified using a BioDrop mLite+ (BioDrop, Cambridge, UK) and standardized to 30 ng/uL before being prepared into sequencing, as described previously.⁴ The extracted DNA was sent to Hangzhou Digital-Micro Biotechnology Co., Ltd. for sequencing. After library construction, WGS was performed on Illumina HiSeq xTen platform using a 2 × 150-bp paired end (PE) configuration. Sequencing reads were trimmed and *de novo* assembled into contigs using the Shovill pipeline (<https://github.com/tseemann/shovill>). Genome analysis was performed in the Center for Genomic Epidemiology (CGE) by uploading the contigs files obtained from the *de novo* assembly of the WGS data.¹² The sequence files were compared with the virulence factor database (VFDB) by ABRICATE software (V.0.8.10) (<https://github.com/tseemann/abricate>) to confirm the virulence factors.

Results

Bacterial Isolates, Antimicrobial Susceptibility Testing and Molecular Epidemiology

From 2015 to 2020, a total of 176 carbapenem-resistant clinical isolates of *Enterobacteriaceae* were recovered from the studied hospital. The bacterial species included *Klebsiella pneumoniae* (73%), *Escherichia coli* (17%), *Klebsiella oxytoca* (8%), *Citrobacter flouidi* (1%), *Raoultella ornithinolytica* (1%). According to the results of the antimicrobial susceptibility test (Table 1), 5 strains of *E. coli* were co-resistant to carbapenem and colistin. By determination of MICs of clinical antibiotics, we showed that all the 5 *E. coli* strains were MDR. *E. coli* co-resistant to carbapenem and colistin had high resistance to antibiotics commonly used in clinic except for amikacin. In addition, the five strains belonged to five sequence types (STs), namely ST131 (n = 1), ST7125 (n = 1), ST10 (n = 1), ST156 (n = 1), and ST457 (n = 1).

Resistance Mechanism of Carbapenem Antimicrobials

Through WGS analysis, a variety of acquired β -lactamase encoding genes were identified in the five *E. coli* strains. EC encoding genes were identified in all 5 *E. coli* isolates with *bla*_{EC-8} as the dominant subtype. The *bla*_{CTX-M-14} (n = 2), *bla*_{CTX-M-65} (n = 1) and *bla*_{CTX-M-199} (n = 1) genes were identified in four of our isolates. AmpC encoding genes was found in one isolates harboring *bla*_{CMY-2}. Furthermore, other β -lactamase encoding genes namely *bla*_{TEM-1} (n = 4) and *bla*_{OXA-10} (n = 1). Carbapenemase encoding genes were authenticated in all isolates through WGS, including *bla*_{NDM-5} (n = 4) and *bla*_{NDM-9} (n = 1). Specific results are shown in Figure 1.

Mechanisms of Colistin Resistance

The MIC of colistin for the five *E. coli* isolates was 4–64 μ g/mL. Four of five *E. coli* isolates were *mcr-1.1* positive. Isolates 19IR1045 was showed the amino acid alterations: Val8Ala in MgrB, the specific results are shown in Figure 1. The *mcr-1.1* coexisted with three or more resistance genes in the *mcr-1.1* positive *E. coli* isolates, demonstrating that the four *mcr-1.1*-positive isolates were all MDR bacteria. In addition, the five *E. coli* strains carried all kinds of efflux pump genes, as shown in Table 2.

Resistance Mechanisms to Other Antimicrobial Agents

Acquired non- β -lactam-resistance genes were observed for aminoglycosides (n = 5), sulfonamides (n = 4), chloramphenicol (n = 4), tetracyclines (n = 3), and macrolides (n = 3). Aminoglycosides encoding genes were *aph(3')-Ia-like* (n = 3), and *aph(6)-Id-like* (n = 3), while sulfonamides resistance was encoded by *sul2* in four isolate. Furthermore, resistance to chloramphenicol and macrolide was encoded by *floR* (n = 3) and *mph(A)* (n = 3) genes, respectively. In addition, three *E. coli* strains carried integrons, being In498, In1411, and In1249 (Table 2).

Table 1 Susceptibility to the Antimicrobial Agents, Clinical Characterization and Phenotypic Detection of *E. coli*

Strain ID	ST Type	Patient's Age	Specimen Source	Isolation Date	MIC (μ g/mL)						
					CAZ	FEP	TZP	IPM	LVX	AMK	COL
IR594	ST7125	2Y6M10D	Pus	2019.05	$\geq 64^R$	16^R	$\geq 128^R$	$\geq 16^R$	2^R	$\leq 2^S$	4^R
IR596	ST10	3Y6M	Pus	2019.06	$\geq 64^R$	$\geq 32^R$	$\geq 128^R$	$\geq 16^R$	$\geq 8^R$	$\leq 2^S$	4^R
19IR1045	ST131	2Y7M	Cyst Fluid	2019.09	$\geq 64^R$	$\geq 32^R$	$\geq 128^R$	$\geq 16^R$	1^I	$\leq 2^S$	$> 64^R$
20IR1127	ST156	2M18D	Pus	2020.10	$\geq 64^R$	$\geq 32^R$	$\geq 128^R$	$\geq 16^R$	$\geq 8^R$	$\leq 2^S$	4^R
20IR1128	ST457	3Y10M	Stool	2020.11	$\geq 64^R$	$\geq 32^R$	$\geq 128^R$	$\geq 16^R$	$\geq 8^R$	$\leq 2^S$	4^R

Note: ^R: Resistant; ^I: Intermediate resistant; ^S: Sensitive.

Abbreviations: ST, sequence type; MIC, minimum inhibitory concentration; Y, year; M, month; D, day; CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin/tazobactam; IPM, imipenem; LVX, levofloxacin; AMK, amikacin; COL, colistin.

Strains	β-lactam resistance												Colistin resistance		Efflux pump gen
	<i>bla</i> NDM-5	<i>bla</i> NDM-9	<i>bla</i> OXA-10	<i>bla</i> CTX-M-14	<i>bla</i> CTX-M-65	<i>bla</i> CTX-M-199	<i>bla</i> TEM-1	<i>bla</i> CMY-2	<i>bla</i> EC	<i>bla</i> EC-5	<i>bla</i> EC-8	<i>bla</i> EC-18	<i>mcr</i> -I.1	<i>mgtB</i>	
IR594															
IR596															
19IR1045															
20IR1127															
20IR1128															

Figure 1 Results of resistance-related mechanisms of 5 carbapenems and colistin co-resistant *Escherichia coli* strains. The black squares in the figure represent positive results, the pale yellow squares represent no mutation results, and the wathet blue squares represent mutations resistance.

Detection of Virulence Genes

Virulence factors expression is more common in some gene-related populations of *E. coli*, which form virulent clones in larger *E. coli* populations. Detection of Virulence Genes The *fimA-I*, *rscB* genes were the most abundant virulence genes detected in the five *E. coli* strains (Table 2). We also detected the virulence genes *fimA-I*, *iroN*, *papB*, *papI*, *rscB*, *fyuA/psn*, *hlyA-D*, *iucA-D*, *iutA*, *papC* and *papG* in our isolates.

Discussion

E. coli can survive in a variety of environment and obtain resistance to various antibiotic by obtaining exogenous resistance genes.¹³ The emergence of MDR *E. coli* poses a huge challenge for effective clinical treatment. Carbapenem is a typical β -lactam antibiotic has the best antibacterial effects at present.¹⁴ Keeping good sensitivity to *Enterobacteriaceae* carrying ESBLs, and is the choice for clinical control of MDR *E. coli* infections. Colistin is an “old” antibiotic, which has been reapplied to the clinic because of its good antibacterial activity against MDR gram-negative rod-shaped bacteria.¹⁵

In this study, 5 carbapenem and colistin co-resistant *E. coli* isolates were screened out from 176 carbapenem-resistant clinical isolates of *Enterobacteriaceae* in a Children's Hospital in China and all 5 *E. coli* isolates showed MDR phenotypes. Through our statistical analysis of WGS data, it can provide a large number of results on resistant isolates, such as serotypes, MLST types, integron and resistant genes. It may take longer and more expensive to obtain all of this data through conventional methods.

All the five *E. coli* strains showed MDR profiles, including resistance to cefepime, ceftazidime, levofloxacin, and piperacillin/tazobactam. These results further reflect the important role of bacterial antibiotic resistance mechanism in clinic. Usually, the carbapenems and colistin were one of the last treatment options in life-threatening multidrug-resistant *Enterobacteriaceae* infections.¹⁶ WGS data show that these isolates contain various determinants of antibiotic resistance, which indicating that carbapenem and colistin co-resistant strains may be selected for any use of antibiotics.

Table 2 Efflux Pump Gene, Integron and Virulence Gene Distribution Characteristics of 5 Strains of Clinically Isolated *E. coli*

Strain ID	ST Type	Resistance Gene	Efflux Pump Gene	Integron	Virulence Gene
IR594	ST7125	<i>bla</i> _{NDM-5} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CMY-2} , <i>bla</i> _{EC-8} , <i>mcr-I.1</i>	<i>emrE</i> , <i>emrD</i> , <i>mdtM</i> , <i>acrF</i>	In498	<i>fimA-I</i> , <i>iroN</i> , <i>iucA-D</i> , <i>iutA</i>
IR596	ST10	<i>bla</i> _{NDM-5} , <i>bla</i> _{CTX-M-14} , <i>bla</i> _{EC} , <i>mcr-I.1</i>	<i>emrE</i> , <i>emrD</i> , <i>mdtM</i> , <i>acrF</i>	In1411	<i>fimA-I</i> , <i>papB</i> , <i>papI</i> , <i>rscB</i>
19IR1045	ST131	<i>bla</i> _{NDM-5} , <i>bla</i> _{CTX-M-14} , <i>bla</i> _{TEM-1} , <i>bla</i> _{EC-5}	<i>emrE</i> , <i>emrD</i> , <i>acrF</i>	/	<i>fimA-I</i> , <i>fyuA/psn</i> , <i>hlyA-D</i> , <i>iucA-D</i> , <i>iutA</i> , <i>papC</i> , <i>papG</i>
20IR1127	ST156	<i>bla</i> _{NDM-5} , <i>bla</i> _{OXA-10} , <i>bla</i> _{CTX-M-65} , <i>bla</i> _{TEM-1} , <i>bla</i> _{EC-18} , <i>mcr-I.1</i>	<i>emrE</i> , <i>emrD</i> , <i>mdtM</i> , <i>acrF</i>	/	<i>fimA-I</i> , <i>rscB</i>
20IR1128	ST457	<i>bla</i> _{NDM-9} , <i>bla</i> _{CTX-M-199} , <i>bla</i> _{TEM-1} , <i>bla</i> _{EC-8} , <i>mcr-I.1</i>	<i>emrE</i> , <i>emrD</i> , <i>mdtM</i> , <i>acrF</i>	In1249	<i>fimA-I</i> , <i>rscB</i>

Although there are not few studies on the resistance of *E. coli* to carbapenem or colistin, the reports on strains isolated from children are not perfect. To the best of our knowledge, this study is the first to report an *E. coli* ST156 strain 20IR1127 in which the genes *bla*_{NDM-5}, *bla*_{CTX-M-65}, *bla*_{OXA-10}, *bla*_{TEM-1} and *mcr1.1* coexist, thereby expounding the molecular characteristics and resistance gene diversity of this strain. In addition, there is an *E. coli* ST457 strain, which carries *bla*_{TEM-1}, *bla*_{NDM-9}, *bla*_{CTX-M-199} and is positive for *mcr1.1* gene. The referential results of these genome sequences are helpful for further comparative analysis of the genomes of *E. coli* strains, and provides genetic background information of the antimicrobial resistance.

Specific mutations in regulators MgrB and the carrying of *mcr* gene are associated with colistin resistance in bacterial, including *K. pneumoniae*, *K. aerogenes*, and *Salmonella enterica*, as well as *P.aeruginosa*, and *A.baumannii*.^{17–19} However, colistin resistance mechanisms in *E. coli* remain to be characterized. A mutation in MgrB was found in only one *E. coli* isolate, and *mcr1.1* gene was found in 4 strains in this study. In the present study, a mutation Val8Ala in MgrB was observed in 19IR1045 isolate. After reading the published studies, the mutation of Val8Ala in MgrB has not been reported in the literature.

According to the literature,^{20,21} ST131 and ST10 *E. coli* is one of the high-risk multidrug-resistant clones with a global distribution and the ability to survive and colonize in a variety of niches. In our study, IR596 and 19IR1045 isolate belongs to ST10 and ST131 colistin-resistant *E. coli*, respectively. Studies have reported that, *E. coli* ST131 is a great model organism to investigate the emergence of superbugs.²⁰ CTX-M-15-producing *E. coli* often belongs to a sequence type called ST131.²⁰ However, our study shows that this strain belongs to CTX-M-14-producing ST131 strain. *E. coli* ST131 also showing high virulence and high antibiotic resistance.²² Indeed, in our study, we also found that ST131 carries more virulence genes and has stronger resistance.

Conclusion

Carbapenem and colistin co-resistant *E. coli* strains cause serious public concern worldwide. To sum up, the main mechanism of carbapenem resistance in this study is production carbapenemase. The plasmid-mediated *mcr* genes contribute to the transfer and occurrence of colistin resistance in 5 *E. coli* strains. In addition, colistin resistance in *E. coli* was related to mutations in regulators by antibiotic selective pressure. Efforts to reduce colistin consumption should be redoubled, to prevent the occurrence of carbapenem and colistin co-resistant *E. coli* strains.

Data Sharing Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; SAMN28858598, SAMN28858599, SAMN28858600, SAMN28858601, SAMN28858602.

Ethics Statement

The studies involving human participants were reviewed and approved by The Research and Ethics committee of The Children's Hospital, Zhejiang University School of Medicine (2021-IRB-031). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author Contributions

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

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Disclosure

The authors declare that they have no competing interests in this work.

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