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

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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 (confering 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. 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, 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*. 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%. 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. The emergence and increase of carbapenem and colistin co-resistant to *E. coli* is an urgent problem to be studied.

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 carbapenem 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).

**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/µL before being prepared into sequencing, as described previously. 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. 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 flodi (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 blaEC-8 as the dominant subtype. The blaCTX-M-14 (n = 2), blaCTX-M-65 (n = 1) and blaCTX-M-199 (n = 1) genes were identified in four of our isolates. AmpC encoding genes was found in one isolates harboring blaCMV-2. Furthermore, other β-lactamase encoding genes namely blaTEM-1 (n = 2), blaCTX-M-65 (n = 1) and blaCTX-M-199 (n = 1). Carbapenemase encoding genes were authenticated in all isolates through WGS, including blaNDM-5 (n = 4) and blaNDM-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 μg/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>
<thead>
<tr>
<th>Strain ID</th>
<th>ST Type</th>
<th>Patient's Age</th>
<th>Specimen Source</th>
<th>Isolation Date</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR594</td>
<td>ST7125</td>
<td>2Y6M10D</td>
<td>Pus</td>
<td>2019.05</td>
<td>≥64R ≥165 ≥128A ≥16B ≥8A ≤2S ≥4A</td>
</tr>
<tr>
<td>IR596</td>
<td>ST10</td>
<td>3Y6M</td>
<td>Pus</td>
<td>2019.06</td>
<td>≥64R ≥32R ≥128A ≥16A ≥28B ≤2S ≥4A</td>
</tr>
<tr>
<td>19IR1045</td>
<td>ST131</td>
<td>2Y7M</td>
<td>Cyst Fluid</td>
<td>2019.09</td>
<td>≥64R ≥32R ≥128A ≥16A ≥28B ≤2S ≥4A</td>
</tr>
<tr>
<td>20IR1127</td>
<td>ST156</td>
<td>2M18D</td>
<td>Pus</td>
<td>2020.10</td>
<td>≥64R ≥32R ≥128A ≥16A ≥28B ≤2S ≥4A</td>
</tr>
<tr>
<td>20IR1128</td>
<td>ST457</td>
<td>3Y10M</td>
<td>Stool</td>
<td>2020.11</td>
<td>≥64R ≥32R ≥128A ≥16A ≥28B ≤2S ≥4A</td>
</tr>
</tbody>
</table>

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.
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, rcsB 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, rcsB, 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. 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. 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.

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. 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>
<thead>
<tr>
<th>Strain ID</th>
<th>ST Type</th>
<th>Resistance Gene</th>
<th>Efflux Pump Gene</th>
<th>Integron</th>
<th>Virulence Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR594</td>
<td>ST7125</td>
<td>bladNDM-5, bladTEM-1, bladCHY-2, bladEC-B, mcr-1.1</td>
<td>emrE, emrD, mdtM, acrF</td>
<td>In498</td>
<td>fimA-I, iroN, iucA-D, iutA</td>
</tr>
<tr>
<td>IR596</td>
<td>ST10</td>
<td>bladNDM-5, bladCTX-M-14, bladEC, mcr-1.1</td>
<td>emrE, emrD, mdtM, acrF</td>
<td>In1411</td>
<td>fimA-I, papB, papl, rcsB</td>
</tr>
<tr>
<td>20IR1127</td>
<td>ST156</td>
<td>bladNDM-5, bladCTX-M-14, bladTEM-1, bladEC-18, mcr-1.1</td>
<td>emrE, emrD, mdtM, acrF</td>
<td>/</td>
<td>fimA-I, rcsB</td>
</tr>
<tr>
<td>20IR1128</td>
<td>ST457</td>
<td>bladNDM-9, bladCTX-M-19B, bladTEM-1, bladEC-B, mcr-1.1</td>
<td>emrE, emrD, mdtM, acrF</td>
<td>In1249</td>
<td>fimA-I, rcsB</td>
</tr>
</tbody>
</table>
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*<sub>NDM-5</sub>, *bla*<sub>CTX-M-65</sub>, *bla*<sub>OXA-10</sub>, *bla*<sub>TEM-1</sub> and *mer1.1* 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*<sub>TEM-1</sub>, *bla*<sub>NDM-9</sub>, *bla*<sub>CTX-M-199</sub> and is positive for *mer1.1* 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 *mer* gene are associated with colistin resistance in bacterial, including *K. pneumoniae*, *K. aerogenes*, and *Salmonella enterica*, as well as *P. aeruginosa*, and *A. baumannii*. However, colistin resistance mechanisms in *E. coli* remain to be characterized. A mutation in MgrB was found in only one *E. coli* isolate, and *mer1.1* 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, 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. CTX-M-15-producing *E. coli* often belongs to a sequence type called ST131. 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. 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 *mer* 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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