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Study on molecular mechanism of carbapenem- and colistin-resistance in *Escherichia coli*

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Abstract

To investigate the drug resistance and molecular mechanism of carbapenem and colistin-resistant *Escherichia coli(E.Coli)* isolated from elderly patients and provide theoretical foundation for clinical anti-infective therapy. 1028 strains of *E.coli* isolated from our hospital were collected. Agar dilution method was used to screen *E. coli* resistant to carbapenem and colistin antibiotics. Broth microdilution method was performed to confirm drug susceptibility and detect susceptibility to 9 commonly used clinical antibiotics. Carbapenem and colistin resistant genes carried by the strains were detected by PCR; Strain ST type was detected by the MLST typing method. Four samples of carbapenem and colistin-resistant *E.coli* were obtained by drug sensitivity test, and all four strains were isolated from elderly patients. It is a multi-drug resistant phenotype resistant to quinolones, cephalosporins, carbapenem and colistin; PCR detection found that DC632 and DC796 carry $bla_{\text{NDM-1}}$ and *mcr-1*, DC721 and DC838 carry $bla_{\text{TEM-1}}$ and *mcr-1*; MLST typing detection found that DC632 and DC721 were ST2 type, DC796 and DC838 were ST44 and ST31, respectively. In conclusion, Four strains of carbapenem- and colistin-resistant *E.coli* were isolated in this study. Although the isolation rate is low, it is still necessary to raise the awareness of clinicians on the rational use of antibiotics.

Keywords: carbapenem; colistin; resistance; mcr-1.

Practical Application: Our findings suggest that more awareness should be raised in clinics regarding the rational use of antimicrobials, monitoring of bacterial resistance, as well as the prevention and control of hospital infections.

1 Introduction

Escherichia coli (*E.coli*) is a common gram-negative pathogen in the clinic, which can cause intestinal infections, urinary tract infections and bloodstream infections (Kaper et al., 2004). Carbapenem are considered one of the most important groups of antimicrobials and are widely used for the treatment for severe infections caused by multidrug-resistant microorganisms (Elshamy & Aboshanab 2020). In recent years, the detection of carbapenem-resistant bacteria has increased at an alarming rate (Nordmann et al., 2011). Emergence and spread of carbapenem resistance have brought great challenges to clinical anti-infective treatment and become a serious public health threat (Elshamy & Aboshanab 2020). Resistance to Carbapenem is mediated by various mechanisms (Elshamy & Aboshanab 2020; Francis & Eric, 2018; Papp-Wallace et al., 2011; Blair et al., 2015; Little et al., 2012): (1) Outer membrane porin-mediated resistance to reduce uptake of Carbapenem. In this mechanism, alterations of porin expression or modifications in porin-encoding genes cause either complete loss or deficiency in the respective porin. (2) Overexpression of efflux pumps, which pump the carbapenem outside the cells. (3) Enzyme-mediated resistance, which is mediated via the acquisition of carbapenemase genes. Carbapenemases catalyze the hydrolysis of Carbapenem and other β-lactam antimicrobials, and thus this resistance mechanism poses a greater threat, as these enzymes may inactivate many other β-lactams (Little et al., 2012; Yang et al., 2009). Moreover, they are encoded by genes carried on transposons, plasmids or other mobile genetic elements, which can be horizontally transferred to other bacterial species (Yang et al., 2009; Tzouvelekis et al., 2012; Stapleton et al., 1999). The nature of the resistance mechanism may influence the dynamics of its spread (Durante-Mangoni et al., 2019). For example, it has been reported that carbapenem resistance in pathogens is conveyed by the New Delhi metallo-beta-lactamase 1 gene (NDM-1), which is encoded on a plasmid and easily transmitted among Gram-negative bacteria, including the human intestinal flora (Durante-Mangoni et al., 2019; Ugwu et al., 2020; Hoang et al., 2013; Fomda et al., 2014). Since the first reported case in 2007, the presence of NDM-1-positive Gram-negative bacteria has been identified across multiple countries and thus carbapenem resistance has become an urgent concern worldwide (Durante-Mangoni et al., 2019; Ugwu et al., 2020; Hoang et al., 2013). However, due to the slow development of new effective antibacterial drugs and limitations in currently available antibacterial therapies, options for clinical anti-infective treatment are increasingly limited. Consequently, clinicians have gradually turned their attention to " new uses for old drugs " and reconsidered the application of old drugs such as polymyxins that were reckoned too toxic for clinical use.

In current study, we focus on colistin (also known as polymyxin E), which is a polymyxin antibiotic first discovered in the late 1940s for the treatment of gram-negative infections (Lim et al., 2010; Nation & Li, 2009). Several years later, clinical use of colistin diminished due to numerous reports of significant nephrotoxicity and neurotoxicity. In recent years, colistin has resurfaced as a

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last-hope treatment option for multidrug-resistant (MDR) Gram-negative bacteria (MDR-GNB), including carbapenem-resistant gram-negative bacteria (Lim et al., 2010; Nation & Li, 2009; El-Sayed Ahmed et al., 2020). Unfortunately, resurgence of colistin is now challenged by global resistance. In particular, with the continuously increasing and unreasonable application of colistin in the clinic, detection and reports of colistin-resistant Escherichia coli have been growing rapidly (Baron et al., 2016). More importantly, with the first discovery of plasmid-mediated colistin resistance gene-*mcr-1* by Chinese scientists in 2015 (Liu et al. 2016), there are more and more reports about colistin-resistant *E.coli* in various regions of the world (Schembri et al., 2015). These cases have greatly attracted the attention of scientists, governments and media, as they brought new challenges to the clinical application of colistin. The rupture of the last line of defense for clinical anti-infection treatment has caused great panic. In the face of the desperate situation of "no medicines available" for anti-infective treatment, it is particularly critical to elucidate the drug resistance characteristics and mechanisms of these strains. Several mechanisms of colistin resistance have been characterized, including intrinsic, mutational, and transferable mechanisms (Aghapour et al., 2019; Gharaibeh & Shatnawi, 2019). A variety of gene mutations have been found to cause colistin resistance by modifying the outer membrane of gram-negative bacteria, which is colistin's site of action (Ge et al., 2016) Although the precise mechanism of resistance remains unclear and seem to be species-dependent, the PmrA-PmrB and PhoP-PhoQ genetic regulatory systems are suggested to play important roles in resistance development (Ge et al., 2016; Quesada et al., 2015). In addition, as colistin is a 'last-line' drug, its dosage must be optimized, while suboptimal dose has been attributed to the spread of resistance.

In clinical practice, strains that are resistant to both Carbapenem and colistin are rare. Therefore, our study focuses on clinical isolation of Carbapenem and colistin in our hospital from 2016 to 2018. Preliminary research on the characteristics and underlying mechanisms of drug-resistant *E. coli* is carried out, which will provide guidance for clinical anti-infection treatment.

2 Materials and methods

2.1 Strains

1,028 strains of *E. coli* were isolated and collected from clinics in our hospital during 2016 to 2018. Strains isolated from the site of the same patient were excluded. All strains were identified by the VITEK_2 Compact automatic microbial analyzer and validated to be *E. coli*. The quality control strains are *E. coli* ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 (both purchased from the Clinical Inspection Center of the Ministry of Health).

2.2 Instruments and reagents

Instruments and reagents used in this study include: VITEK® 2 Compact (BioMérieux, France), Thermocycler (Bio-Rad, U.S), Bacterial Genomic DNA Miniprep Kit (Axyprep, U.S).

2.3 Strain screening and isolation

According to the CLSI (Papp-Wallace et al., 2011) standard, single colony of *E.coli* or the quality control strain ATCC25922 was picked and inoculated on the blood agar plate. Turbidity of growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units using sterile saline. Agar dilution method was used to screen for *E.coli* strain with resistance to both carbapenem and colistin. Minimal inhibitory concentration (MIC) of colistin refers to the standard of European Committee Antimicrobial Susceptibility Testing (EUCAST): ≤2 μg/mL is categorized as susceptible, >2 μg/mL is categorized as resistant. MIC standard of carbapenem is referred to Clinical Laboratory Standardization Committee (CLSI): imipenem>4μg/mL is resistant, ≤2 μg/mL is susceptible.

2.4 Resistance analysis

Carbapenem and colistin-resistant *E. coli* were tested for the minimal inhibitory concentration (MIC) by broth dilution method for nine different antimicrobial drugs, including: Ceftazidime (CAZ), Cefotaxime (CTX), Ciprofloxacin (CIP), Amikacin (AMK), Gentamicin (GEN), Levofloxacin (LEV), Imipenem (IMP), American Ropenem (MEM), fosfomycin (FOS) and colistin (COL). Break point is determined according to the standard of the Clinical Laboratory Standardization Committee (CLSI) (Papp-Wallace et al., 2011).

2.5 Resistance analysis

Genomic DNA from Carbapenem and colistin-resistant *E. coli* was purified using DNA purification kit. Plasmid-mediated colistin resistance gene *mcr-1*, chromosome-mediated resistance gene pmrAB, carbapenem resistance gene and quinolone resistance gene are amplified by polymerase chain reaction (PCR) and primer sequence are designed according to literature (Kaper et al., 2004; Elshamy & Aboshanab, 2020; Blair et al., 2015; Little et al., 2012). Detailed conditions for PCR reaction are: pre-denaturation at 94°C for 15min, followed by 35 cycles of deformation at 94°C for 1min, annealing for 30s, extension at 72 °C for 30s, and final extension reaction at 72 °C for 10min. The PCR products were subjected to 1% agarose gel electrophoresis, GelRed staining, and gel imaging to observe results. The positive products were sent to Beijing Genomics Institute (BGI) for sequencing, and the sequences were compared and analyzed by Blast and NCBI database.

2.6 Bioinformatics and data and statistical analyses

The workflow of Multilocus sequence typing (MLST) analysis include: Carbapenem and colistin-resistant *E. coli* genome extraction → PCR amplification of 8 housekeeping genes (*dinB, icdA, pabB, polB, putP, trpA, trpB, uidA*) → PCR product sequencing → Comparison to MLST database and database of the Centers for Disease Control and Prevention (2021).

3 Results

3.1 Isolation of Carbapenem and colistin-resistant E. coli

Through a screening and validation of 1028 *E. coli* strains isolated from our hospital, a total of four strains showing simultaneous resistance to both carbapenem and colistin were obtained. These strains were isolated from two urine specimens,

one sputum specimen and one blood specimen. Detained results are shown in Table 1 below.

3.2 Analysis for carbapenem and colistin resistance

Four Carbapenem and colistin-resistant *E. coli* strains were screened for susceptibility to commonly used antimicrobial drug in clinics. Interestingly, we found that all four strains exhibited multiple resistance; they were resistant to both quinolones and cephalosporins simultaneously, are shown in Table 2.

3.3 Study on the mechanism of carbapenem and colistin resistance

In order to elucidate the underlying mechanism for the resistance to carbapenem and colistin, we performed PCR reactions to detect and analyze the presence of carbapenem and colistin resistant genes in these strains. As showed in Table.3, we found that DC632 and DC796 carry *blaNDM-1* and *mcr-1*, while DC721 and DC838 carry *blaTEM-1* and *mcr-1*.

3.4 MLST typing of carbapenem and colistin resistant E. coli strains

In addition to analysis of resistance gens, we further characterize these carbapenem and colistin resistant *E. coli* strains by performing a multilocus sequence typing test (MLST), as shown in Table 3. According to our typing test, DC632 and DC721 were identified as ST2 types, while DC796 and DC838 were ST44 and ST31 types, respectively.

4 Discussion

With the extensive application of carbapenem antibacterial drugs in clinics, the detection rate of carbapenem-resistant *E.coli* is elevating rapidly, which brings tremendous challenges to clinical treatment and infection control (Tzouvelekis et al., 2012). Polymyxin is a cationic antibacterial peptide that works through binding with lipopolysaccharide on the outer membrane of gram-negative bacteria to damage bacterial cell wall (Nordmann et al., 2011). Polymyxin is commonly used as the final line of defense against sever infections caused by carbapenem-resistant gram-negative bacteria. However, in recent years, with irrational clinical use and extensive application of polymyxin in breeding industry, the detection of polymyxin-resistant *E.coli* has progressively increased, posing a serious challenge to clinical anti-infection treatment.

In this study, 1028 strains of *E.coli* isolated from clinics in our hospital during 2016 to 2018 were screened for resistance to carbapenem and colistin, and a total of 4 strains were obtained. Interestingly, we found that all four strains were isolated from elderly patients. The rationale behind this observation may be explained by factors including increased underlying health conditions, weaker immunity, and frequent hospitalization with colonization of carbapenem Enterobacter in elderly patients (Lerner et al., 2015). Moreover, we speculate that polymyxin resistance is potentially obtained through widespread accumulation of resistance through food chains (Huang et al., 2017). In addition, with the four Carbapenem and colistin-resistant *E.coli* strains from our screening, we carried out susceptibility test of commonly used antibacterial

Table 1. Presence of colistin resistant *E. coli* isolates from 2016 to 2018.

Year	No. of strains tested	No. of positive strains	Positive rate(%)
2016	298	1	0.34%
2017	351	1	0.28%
2018	379	2	0.53%

Table 2. MICs (μg/mL) of colistin resistant *E. coli* isolates.

Strain	MIC(μg/mL)									
	IMP	MEM	COL	AMK	CIP	LEV	CAZ	CTX	FOS	GEN
DC632	4	8	4	2	>16	16	≥64	≥32	8	4
DC721	8	8	8	4	>16	8	≥64	≥32	8	8
DC796	8	16	16	2	>16	16	32	≥32	64	2
DC838	4	8	8	16	>16	16	32	≥32	64	>64
ATCC25922	0.125	0.015	0.5.	1	0.008	0.03	0.25	0.06	1	0.5
ATCC27853	2	0.5	1	2	0.5	1	2	16	2	1

Table 3. Carbapenem and colistin-resistant *E. coli* strains carry resistance genes and MLST typing.

Strain	Resistance gene	MLST typing
DC632	bla_{NDM-1}、*mcr-1*、*qnrA*	ST2
DC721	bla_{TEM-1}、*mcr-1*、*qnrA*	ST2
DC796	bla_{NDM-1}、*mcr-1*、*qnrD*	ST31
DC838	bla_{TEM-1}、*mcr-1*、aac (6')-Ib-cr	ST31

drugs in clinic. Notably, we found that they were resistant to quinolones and cephalosporins at the same time, and all four strains showed multi-drug resistance. This result further prompted us to pay attention to the gained resistance of colistin and Carbapenem. Greater emphasis must be placed on the rational and precautions application of antibacterial drugs, in order to prevent strains from forming extensive or even pan-drug resistance, which will eventually result in clinically no drugs available (Delgado-Blas et al., 2016).

To further understand the underlying mechanism of resistance, we performed genotypic studies and examined several genes that have been linked to the development of carbapenem and colistin resistance. MCR-1 is a phosphoethanolamine transferase coded by *mcr-1* and confers resistance to colistin by transferring phosphoethanolamine to lipid A (Mediavilla et al., 2016). Gene *mcr-1* was identified as the first transferable plasmid-mediated colistin resistance gene, which has been reported in *E. coli* isolates from animals, food, and patients worldwide (Wang et al., 2017; Zhang et al., 2019). The presence of *mcr-1* is in transferable plasmids further exacerbate the spread of colistin resistance mediated by mcr-1. Moreover, *blaNDM-1* is also reported as a plasmid-mediated carbapenem resistance gene; NDM-1 and its pathogen-producing variants prevail in different countries, which may potentially accelerate the spread of carbapenem resistance (Murugan et al., 2019; Yong et al., 2009; Zhong et al., 2016; Uchida et al., 2018). More importantly, co-harboring of *mcr-1* and *blaNDM* has been reported in *E. coli* and other members of the family Enterobacteriaceae from animals, as well as patients with peritonitis, urinary tract infections, mostly in China (Uchida et al., 2018). In addition, TEM-1 beta-lactamase is well-characterized as one of the most widespread enzymes to confer plasmid-mediated β-lactam antibiotic resistance to gram-negative bacteria (Sideraki et al., 2001; Delmani et al., 2017). In present study, our screening and analysis of carbapenem and colistin resistance genes showed that strain DC632 and DC796 carry both *blaNDM-1* and *mcr-1*, while DC721 and DC838 harbor both *blaTEM-1* and *mcr-1*As our data identified the co-harboring of resistance genes in clinical isolates of *E.coli*, more attention should be paid to the strains carrying both *blaNDM-1* and *mcr-1* genes to prevent the widespread of drug resistance in clinics (Yong et al., 2009). Furthermore, our results of MLST analysis showed that the two strains (DC632 and DC721) exhibited the same typing of ST2, suggesting potential clonal transmission of these two strains (European Committee on Antimicrobial Susceptibility Testing. 2015). Therefore, in order to minimize the undesirable outcomes of resistance and further improve infection control, careful monitoring of hospital infection and routine surveillance for the emergence and spread of the resistant strains are required (Logan & Weinstein, 2017).

In summary, the four *Escherichia coli* strains resistant to both Carbapenem and colistin in this study are mainly related to resistance genes such as blaNDM-1, blaTEM-1 and mcr-1. More importantly, our findings suggest that more awareness should be raised in clinics regarding the rational use of antimicrobials, monitoring of bacterial resistance, as well as the prevention and control of hospital infections.

Ethical approval

This study received approval and ethical clearance from the Medical Ethics Committee of Yantai Yuhuangding Hospitall.

Conflict of interest

The authors declare that they have no competing interests.

Availability of data and material

The data used to support the finding of this study are included within the article.

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