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ORIGINAL RESEARCH

Resistance and Heteroresistance to Colistin in *Escherichia coli* Isolates from Wenzhou, China

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Background: Colistin is being administered as last-line therapy for patients that have failed to respond to other available antibiotics that are active against *Escherichia coli*. The underlying mechanisms of colistin resistance and heteroresistance remain largely uncharacterized. The present study investigated the mechanisms of resistance and heteroresistance to colistin in *Escherichia coli* isolates from Wenzhou, China.

Materials and Methods: Colistin resistance was detected by the broth microdilution method (BMD). Colistin heteroresistance was determined by population analysis profiles (PAPs). The polymerase chain reaction (PCR) was conducted to detect *mcr-1, mcr-2, mcr-3, pmrA, pmrB, phoP, phoQ* and *mgrB,* and quantitative real-time PCR (qRT-PCR) was used to determine the expression levels of *mcr-1, pmrC, pmrA* and *pmrB*. Lipid A characterization was conducted by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).

Results: 0.69% (2/291) of *Escherichia coli* strains were resistant to colistin, whereas the heteroresistance rate reached 1.37% (4/291). *mcr-1*, the mobile colistin-resistance gene, was present in the two resistant isolates. The substitutions in PmrB were detected in the two heteroresistant isolates. The transcripts levels of the *pmrCAB* operon were upregulated in two of the heteroresistant isolates. carbonylcyanide m-chlorophenylhydrazone (CCCP) was able to reverse colistin resistance of all isolates tested and exhibited a significantly higher effect on colistin-heteroresistant isolates. MALDI-TOF MS indicated that the additional phosphoethanolamine (PEtn) moieties in lipid A profiles were present both in colistin-resistant and heteroresistant isolates.

Conclusion: The present study was the first to investigate the differential mechanisms between colistin resistance and heteroresistance. The development of colistin heteroresistance should be addressed in future clinical surveillance.

Keywords: *Escherichia coli*, colistin, *mcr-1*, heteroresistance, lipid A

Introduction

Escherichia coli (*E. coli*) is one of the most commonly isolated microorganisms in clinical specimens, which is responsible for various nosocomial infections, such as meningitis, urinary tract infections (UTIs), wound infections and bloodstream infections.[1–3] Treatment of multidrug-resistant (MDR) *E. coli* infections has become a serious clinical issue, notably with the emergence of resistance to last-resort antibiotics such as colistin.[4]

As a cationic antimicrobial polypeptide, colistin exerts antibacterial activity via interacting with the lipid A moiety of lipopolysaccharide (LPS) and subsequently disrupting the outer membrane of Gram-negative bacteria.[5,6] Despite the potential of colistin as a high-efficient antimicrobial agent, its massive use in clinical

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therapeutics and as a feed additive has led to the development of colistin resistance in *E. coli*.[7,8]

To date, the most common reported mechanisms for colistin resistance in *E. coli* have involved the plasmid-borne mobile colistin resistance (*mcr*) gene, which was newly discovered in 2016 and denoted as *mcr-1*.[9] Subsequently, several amino acid variants have been described, such as *mcr-2* and *mcr-3*.[10,11] This transferable mechanism of colistin plasmid-borne resistance could aggravate and contribute to the spread of resistance. In addition, chromosomally encoded systems, such as PhoPQ and PmrAB two-component regulatory systems (TCS) have been reported sporadically.[7]

Heteroresistance to colistin has been reported to be significantly increased in Gram-negative bacteria.[12] This has been shown by a phenotypic characteristic demonstrating the presence of colistin-resistant subpopulations among a susceptible population.[13] Several mechanisms of colistin heteroresistance have been proposed, including the activation of PmrAB and PhoPQ TCSs (*Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Enterobacter cloacae*),[14–17] overexpression of the efflux pumps (*Acinetobacter baumannii, Enterobacter asburiae* and *Enterobacter cloacae*),[18,19] biofilm formation (*Klebsiella pneumoniae*)[20] and putrescine/YceI communication (*Burkholderia cenocepacia*).[21] However, only sporadic cases of colistin heteroresistance have been reported in *E. coli*,[22] let alone the investigation of their mechanisms of heteroresistance against this compound.

The aim of our present study was to determine and compare the mechanisms that are responsible for resistance and heteroresistance to colistin in *E coli* strains isolated from a Chinese teaching hospital.

Materials and Methods

Bacterial Strains

A total of 291 non-duplicated *E. coli* clinical isolates were recovered from the First Affiliated Hospital of Wenzhou Medical University, China in 2018. Each isolate represented a single sample from one patient. The isolates were identified as *E. coli* by VITEK® MS automated system (BioMérieux, Hazelwood, MO, USA). The MICs of colistin were determined by the broth microdilution in cation-adjusted Mueller-Hinton Broth (CAMHB).[23] The MIC breakpoint of colistin for *E. coli* was interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2019)[24] (susceptible, ≤2 mg/L; resistant, >2 mg/L). The *E. coli* ATCC 25,922 served as the quality control for susceptibility testing.

Population Analysis Profiles (PAPs)

PAPs are used as the reference method to define antibiotic hetero-resistance.[15] The analysis was performed among colistin-susceptible isolates. Bacterial cultures were grown overnight to the log-phase and subsequently plated using serial dilutions on Luria-Bertani (LB) agar with or without various concentrations of colistin (0, 0.25, 0.5, 1, 2, 4, 8 and 16 mg/L). The plates were incubated at 37°C and the Colony-Forming Units (CFU) were enumerated following 48h. The limit of detection was 20 CFU/mL. Colistin heteroresistance was defined as a colistin-susceptible isolate (MIC ≤2 mg/L) with subpopulations growing in the presence of ≥2 mg/L colistin.[25] The percentage of colistin resistance was calculated as the number of bacterial colonies that grew on colistin plates divided by the number of bacteria that grew on LB broth without drug. For each isolate, a single colony was selected from the highest antibiotic concentration and the colistin MIC was reassessed following serial passaging on antibiotic-free medium to evaluate the stability of the hetero-resistant phenotype. Cultures with resistant or susceptible subpopulations were isolated from the highest colistin concentration or drug-free medium separately for further studies.

Antimicrobial Susceptibility Test

The MICs of clinical routine antimicrobial agents, including aztreonam (ATM), ceftazidime (CAZ), fosfomycin (FOS), gentamicin (GEN), levofloxacin (LEV), meropenem (MEM), amikacin (AMK), ciprofloxacin (CIP), imipenem (IMP) and cefotaxime (CTX) were determined using the agar dilution method (BMD) in accordance with the guidelines of CLSI 2019.[26] To examine the effects of efflux pump on colistin resistance and heteroresistance, MICs were measured via the broth microdilution method (BMD) by adding 10 mg/L of carbonylcyanide m-chlorophenylhydrazone (CCCP) into CAMHB.[27]

Polymerase Chain Reaction (PCR) and Sequencing

Whole-cell DNA samples of colistin-resistant and -heteroresistant isolates were extracted using the Biospin Bacterial Genomic DNA Extraction kit (Bioflux, Tokyo, Japan) according to the manufacturer's instructions. The genes of *mcr-1, mcr-2, mcr-3, pmrA, pmrB, phoP, phoQ* and *mgrB* among the *E. coli* strains were investigated by PCR and

Table 1 PCR Primers Used in This Study

Gene	Sequence (5′→3′)	Amplicon Size (bp)
Primers for sequencing		
mcr-1	F: GCTCGGTCAGTCCGTTTG R: GAATGCGGTGCGGTCTTT	1626[28]
mcr-2	F: TGTTGCTTGTGCCGATTGGA R: AGATGGTATTGTTGGTTGCTG	1617[29]
mcr-3	F: AAATAAAAATTGTTCCGCTTATG R: AATGGAGATCCCCGTTTTT	929[30]
pmrA	F: AGTTTTCCTCATTCGCGACCA R: TACCAGGCTGCGGATGATATTCT	714[31]
pmrB	F: GGATGGCCTGATGTGACGCTGTC R: GCGCGGCTTTGGCTATA	1312[31]
phoP	F: GCCAGTACCGCCAGCTTAA R: CTCGCCACGTAACAGCCGAA	1798[31]
phoQ	F: GGCACAATATCCCCAAGAAGT R: ATCCACAGGCTGGTATCTGCA	1595[31]
mgrB	F: CACGAATATCGACATAGTTAG R: TATTCTACCACTGCTGGAGAG	275[31]
Primers for qRT-PCR		
gapA	F: CGTTAAAGGCGCTAACTTCG R: ACGGTGGTCATCAGACCTTC	116[7]
mcr-1	F: GCAGCATACTTCTGTGTGGTAC R: ACAAAGCCGAGATTGTCCGCG	145[33]
pmrC	F: CTGATGACGCCACGAATG R: TTGTAGAACAGTGCGGCAAC	134[7]
pmrA	F: CCTTTTGCGCTGGAAGAG R: TGGGCGTCAGAATCAACTC	156[7]
pmrB	F: TCCCCTCGTATGACGAACTC R: TCATAATGTTGCTGCCTTGC	112[7]

the primers are described in Table 1.[28–31] The amplicons of *mcr-1, mcr-2, mcr-3, pmrA, pmrB, phoP* and *phoQ* and *mgrB* were sequenced by Shanghai BGI Technology Co., Ltd. and subsequently analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of resistant isolates were compared with those of ATCC 25,922, while the comparison was carried out between susceptible and resistant subpopulations in heteroresistant isolates.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA samples from heteroresistant isolates (including susceptible and resistant subpopulations) were extracted from the log-phase bacterial inoculum using the Bacterial RNA Miniprep Kit (Biomiga, Shanghai, China). An aliquot of RNA from each isolate was subjected to cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, Shanghai, China). The expression levels of *pmrC, pmrA* and *pmrB* were assessed by the qRT-PCR assay.[7] The *gapA* housekeeping gene was used as a calibrator. The expression levels of the three genes in the heteroresistant isolates were performed by comparing their susceptible and resistant subpopulations. The ΔΔCt method was used to quantify gene expression. The experiments for each gene were conducted in triplicate.

In order to evaluate the impact of CCCP on colistin resistance and heteroresistance, *mcr-1* gene transcription and *pmrC* gene expression were quantified in colistin-resistant and colistin-heteroresistant isolates, respectively. RNA was extracted from LB broth cultures containing 2 mg/L colistin +10 mg/L CCCP, as described previously.[32] The RNA was used to quantify gene expression as previously mentioned. The primers used in the present study are listed in Table 1.[7,33]

Lipid A Isolation from Whole Cells

Lipid A was isolated by an optimized large-scale mild acid hydrolysis-based protocol.[34] Overnight cultures (200 mL at 37°C) in LB broth were harvested via 3220×*g* for 30min. Bacterial pellets were washed by single-phase Bligh–Dyer mixture (chloroform/methanol/water; 1:2:0.8, v/v) and centrifuged at 3220×*g* for 15min. The LPS pellets were suspended with sodium acetate buffer (50 mM, PH 4.5) and incubated at 100°C for 30–45min. The reactions were converted into a two-phase Bligh–Dyer mixture (chloroform/methanol/water; 1:1:0.9, v/v) and centrifuged at 3220×*g* for 15min. The lower phases were removed to clean tubes and dried using rotary evaporation. The dried samples contained whole-cell extracts of lipid A.

Lipid A Characterization by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

Dried lipid A samples were resuspended in 100 μL chloroform/methanol mixture (1:1, v/v). A total of 3 μL DHB matrix (20 mg/mL in TA30) were mixed with 3 μL lipid A. Aliquots of mixture were spotted directly onto the well of the MALDI-TOF plate (Ground Steel). The mass spectra were recorded for optimal ion signals in negative mode using a Brukerautoflex MALDI-TOF mass spectrometer

(BrukerDaltonics Inc., Billerica, MA, USA). The data were acquired and processed by flexControl and flexAnalysis 3.4 (BrukerDaltonics Inc.).

Statistical Analysis

The differences in the expression levels of each gene of interest were tested using the Student's *t*-test. For all analyses, a *P* value lower than 0.05 ($P<0.05$) was used for significant differences. All statistical calculations were conducted with the SPSS v.22.0 software (SPSS Inc., Chicago, IL, USA).

Results

Antibiotic Susceptibility Profiles of Colistin-Resistant and Heteroresistant Isolates

Of the 291 *E. coli* strains, two isolates (DC 8277 and DC 8313) were resistant to colistin with Minimum inhibitory concentration (MIC) of 4 mg/L and 8 mg/L, respectively. PAPs were used as a standard method for determining colistin heteroresistance. Four isolates (DC 8243, DC 8253, DC8446 and DC8471) were detected out of colistin-susceptible *E. coli* strains. The results indicated that the growth of subpopulations with 16- to 64-fold dilutions exhibited higher colistin MIC than their parental populations. The frequency of heteroresistant subpopulations ranged from 4.0×10^{-7} to 4.0×10^{-6}. The MICs of colistin against the resistant subpopulations did not change following 1 week of subculturing in antibiotic-free medium. All colistin-resistant isolates and two of four colistin-heteroresistant isolates exhibited multidrug-resistance (Table 2).

Analysis of Mobile Colistin-Resistance (*mcr*) Mechanism

The *mcr* genes in the colistin-resistant and heteroresistant isolates were analyzed by PCR. Both of the two resistant isolates carried the *mcr-1* gene, which was not present in any of the heteroresistant isolates, while *mcr-2* and *mcr-3* were not detected in all studied isolates (Table 3).

Analysis of TCS Mechanism-Involvement in Colistin (Hetero)Resistance

In the present study, *pmrA, pmrB, phoP, phoQ* and *mgrB* were sequenced in DNA samples extracted from two resistant isolates and four heteroresistant isolates. No mutation was detected in the two resistant isolates. In the heteroresistant group, we observed a mutation of PmrB (L14R) in DC 8243 and PmrB (P95L) in DC 8471 (Table 3).

To connect the molecular genetic mutations with the transcriptional expression level, the *pmrC, pmrA* and *pmrB* expression levels of four colistin-heteroresistant isolates were analyzed. The results are presented in Figure 1. Compared to its native isolate, DC 8243 indicated significantly higher relative expression levels in *pmrC, pmrA* and *pmrB*, which were upregulated by 123.99-, 16.93- and 4.64-fold, respectively ($P<0.05$). In DC 8253, the expression levels of *pmrC, pmrA* and *pmrB* were slightly increased by 5.29- ($P<0.05$), 3.48- and 2.05-fold. In the isolates DC 8446 and DC 8471, the increment of the expression levels was not found.

The Effect of CCCP on Colistin (Hetero) Resistance

We tested the effects of the efflux pump inhibitor CCCP on both colistin-resistant and heteroresistant *E. coli* isolates. All strains were found to rescue colistin susceptibility (MIC ranged from 0.06 to 1 mg/L) following exposure to CCCP at a concentration of 10 mg/L (Table 2).

Subsequently, the effects of CCCP on colistin-related gene expression were analyzed on two resistant isolates and four heteroresistant isolates. In the colistin-resistant group, the addition of 2mg/L colistin + 10 mg/L CCCP led to a 0.75-fold and 0.49-fold decrease in the expression levels of the *mcr-1* gene with regard to the isolate DC 8277 and the isolate DC 8313, respectively (Figure 2). However, the results were not statistically significant ($P>0.05$). In the colistin-heteroresistant group, the expression levels of *pmrC* were considered an indicator and all the isolates exhibited significantly decreased expression levels, ranging from 5- to 1000-fold ($P<0.05$) (Figure 3).

Lipid A Modifications in Colistin-Resistant and Heteroresistant Isolates

Lipid A was extracted from (hetero)resistant strains and profiled using MALDI-TOF MS to analyze modifications associated with observed genetic alterations. The mass spectrum for lipid A from the colistin-susceptible and reference strain ATCC 25,922 indicated major peaks at the mass/charge ratios (*m*/*z*) of 1796, which have been known to correspond to the hexa-acyl diphosphoryl lipid A.

Compared with the mass assigned to native lipid A, the additional peak was observed at the *m*/*z* of 1919 on

Table 2 Susceptibility Characteristics of the Six *Escherichia coli* Isolates Studied

Isolate	Broth MIC (mg/L)	Highest Concentration of Growth in PAPs (mg/L)	Proportion of Resistant Subpopulations	Resistant Colonies MIC After 1 Week Daily Passages onto Colistin-Free Medium (mg/L)	Population Analysis Profiling	The MICs of Clinical Routine Antimicrobial Agents (mg/L) [a]									
						CAZ	FOS	GEN	LEV	MEM	AMK	CIP	IPM	CTX	COL +CCCP
DC8277	4	8	NA	NA	resistant	2	1	16	≥8	0.06	1	≥8	0.5	32	0.5
DC8313	8	8	NA	NA	resistant	32	1	1	≥8	0.015	2	≥8	0.25	8	1
DC8243	0.25	16	2.2×10^{-6}	16	Hetero-resistant	8	2	1	0.25	0.03	2	0.125	0.25	16	0.06
DC8253	0.25	16	4.0×10^{-7}	8	Hetero-resistant	1	2	1	0.015	0.06	2	0.125	0.25	0.06	0.06
DC8446	0.5	8	4.0×10^{-7}	8	Hetero-resistant	16	2	64	4	0.06	8	≥8	0.125	≥128	0.125
DC8471	0.5	8	4.0×10^{-6}	8	Hetero-resistant	16	1	64	0.5	0.06	2	1	0.25	≥128	0.25

Notes: [a]For heteroresistant isolates, the objects of MICs detection are resistant subpopulations selected from PAPs.

Abbreviations: CAZ, ceftazidime; FOS, fosfomycin; Gen, gentamicin; LEV, levofloxacin; MEM, meropenem; AMK, amikacin; CIP, ciprofloxacin; IPM imipenem; CTX, cefotaxime; COL, colistin; CCCP, carbonylcyanide m-chlorophenylhydrazone.

Table 3 Analysis of Mobile Colistin Resistance (*mcr*) Genes and TCS Regulatory Pathways in (Hetero)resistant *E. coli* Isolates

Isolates	Mobile Colistin Genes (*mcr*) [a]			TCS Regulatory Pathways [b]				
	mcr-1	*mcr-2*	*mcr-3*	*pmrA*	*pmrB*	*phoP*	*phoQ*	*mgrB*
Colistin resistant								
DC 8277	+	–	–	/	/	/	/	/
DC 8313	+	–	–	/	/	/	/	/
Colistin heteroresistant								
DC 8243	–	–	–	/	L14R	/	/	/
DC 8253	–	–	–	/	/	/	/	/
DC 8446	–	–	–	/	/	/	/	/
DC 8471	–	–	–	/	P95L	/		/

Notes: In colistin-resistant group, the sequences were compared with the colistin-susceptible *Escherichia coli* ATCC 25,922. In colistin-heteroresistant group, the comparison was carried on between susceptible and resistant subpopulations. [a] +, *mcr*-positive. -, *mcr*-negative. [b]/, mutation was not detected. L, Leu, leucine; R, Arg, arginine; P, Pro, proline. L14R, corresponding to the 14th amino acid substitution of *pmrB* from Leu to Arg; P95L, corresponding to the 95th amino acid substitution of *pmrB* from Pro to Leu.

MALDI-TOF spectra obtained from all two resistant isolates and four heteroresistant isolates, which was associated with an addition of phosphoethanolamine (PEtn) moiety (*m/z*123 mass units) at the C-1 of the native lipid A (Figure 4A). The colistin-heteroresistant isolates, DC 8243 and DC 8253 demonstrated the presence of ions at *m/z* 2035 and 2158, which resulted from a palmitoylation (*m/z*239 mass units) at the C-2 position of the structures identified with *m/z* of 1796 and 1919. An additional peak at *m/z* 2066 was observed for the isolate DC 8446. This peak was assigned to the addition of a 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety (*m/z*131 mass units) onto the phosphate group at position C'-4 of native lipid A with concomitant addition of the hydroxylation group (*m/z*16 mass units) at C'-2 (Figure 4B). These findings were consistent with previous studies.[35] The lipid A structures and the corresponding *m/z* values of these signature ions are shown in Figure 5.

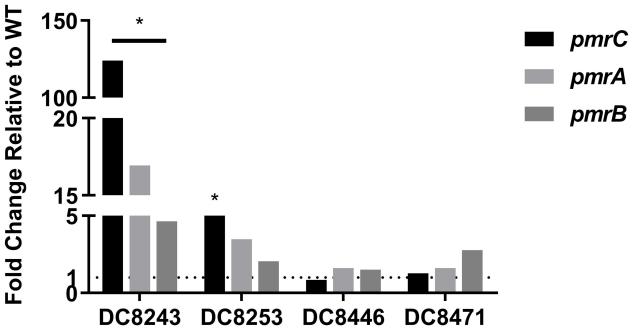


Figure 1 Expression levels of regulatory genes in colistin-heteroresistant isolates. **P*<0.05; WT, native isolates.

Discussion

Colistin has been proposed as a last-line antibiotic for the treatment of MDR Gram-negative pathogens infections. Despite its superior bacterial killing, colistin resistance and heteroresistance have gained global attention and pose a new threat to public health.[36,37] In the present study, we presented the first report of the mechanisms of

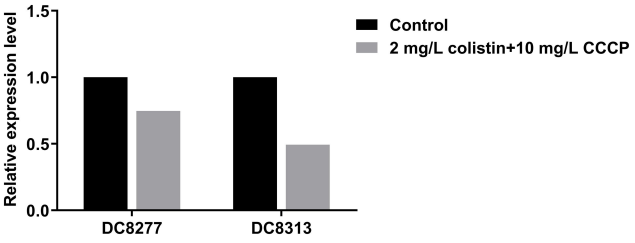


Figure 2 *Mcr-1* gene expression in colistin-resistant *E. coli* in the absence of antibiotics and following 2 mg/L colistin +10 mg/L CCCP.

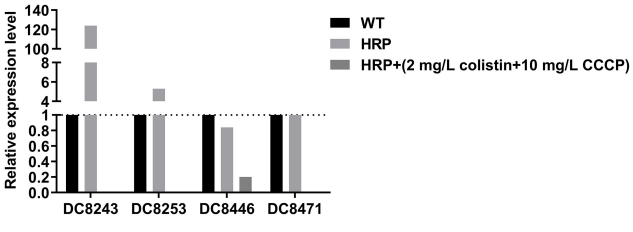


Figure 3 *pmrC* gene expression in colistin-heteroresistant *E. coli* in the absence of antibiotics and following 2 mg/L colistin +10 mg/L CCCP; WT, native isolates; HRP, heteroresistant subpopulations.

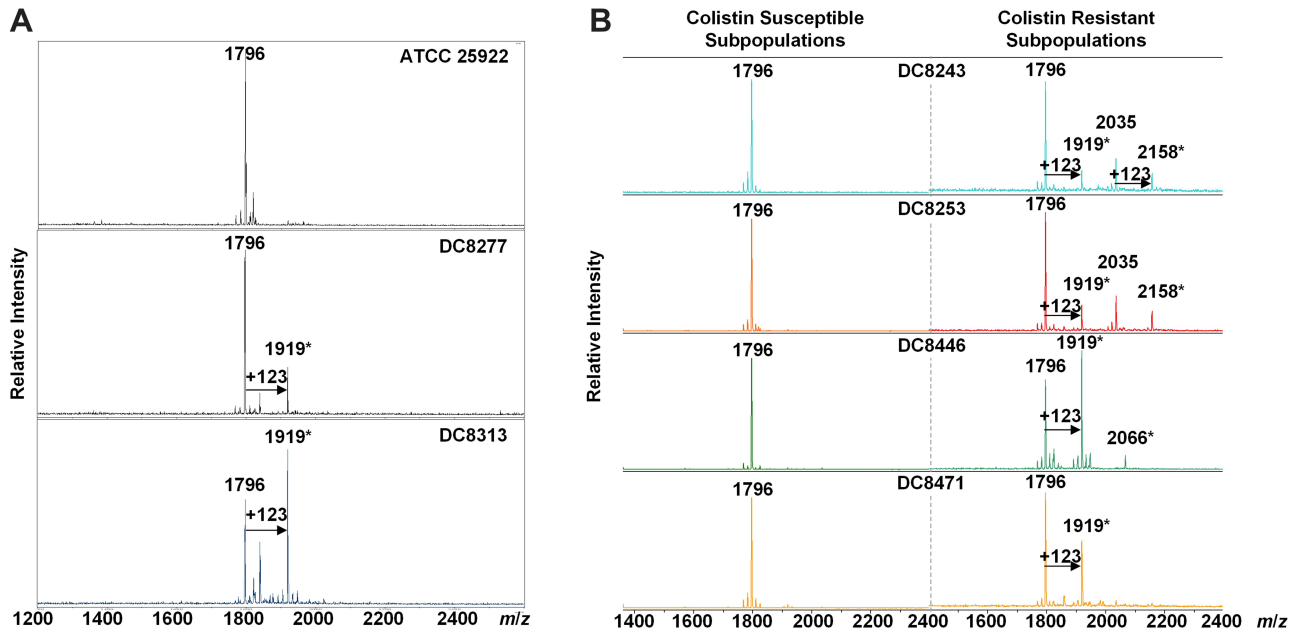


Figure 4 MALDI-TOF of *E. coli* with differential colistin susceptibility. (**A**) The MS profiles of colistin-resistant isolates. *, Lipid A modification; (**B**) The MS profiles of colistin-heteroresistant isolates. *Lipid A modification.

resistance and heteroresistance to colistin in *E. coli* clinical isolates.

Two colistin-resistant isolates and four colistin-heteroresistant isolates were acquired from *E. coli* clinical isolates in 2018 by antimicrobial sensitivity testing and PAP, respectively. The resistance rate of *E. coli* to colistin was 0.69% (2/291), which was slightly higher than that of 2002–2016 noted in our previous study (0.35%, 12/3434).[38] Furthermore, the rate of heteroresistance was 1.37% (4/291), which was considerably lower than that noted in other reports.[22] The reasons for the differences may be attributed to the sample size, single location and the detection methods used in different regions of the study.[39] The four heteroresistant isolates exhibited MICs for colistin at 8–16 mg/L, two of which indicated MDR phenotypes (Table 2).

The mechanisms of colistin resistance were mainly due to chromosomal and plasmid-mediated resistance. In the *E. coli*, the presence of the plasmid-mediated *mcr* gene is the most common mechanism of resistance, notably for the case of *mcr-1*.[40–44] We found that both of the resistant isolates possessed *mcr-1* (Table 3), which was consistent with previous findings.[40–44]

By contrast, colistin-heteroresistant strains were more common than resistant strains.[45] El- Halfawy et al demonstrated that heteroresistance was an emerging field that required further investigation.[46] Despite certain reports that have been published regarding heteroresistance in the past years, the perception of colistin heteroresistance in *E. coli* is relatively limited and remains undiscovered. Therefore, we examined the possible mechanisms by analyzing the role of PmrAB and PhoPQ TCS. The results indicated that the strains DC 8243 and DC 8471 exhibited mutations in *pmrB* and led to amino acid substitutions (L14R and P95L in PmrB, respectively)(Table 3). Mutations in the *pmrB* gene can activate PmrAB TCS, which causes the upregulation of the *pmrCAB* operon.[47] Additional studies indicated that the expression levels of *pmrC, pmrA* and *pmrB* in strain DC 8243 were significantly upregulated ($P<0.05$). In comparison, strain DC 8471 did not show the corresponding changes, indicating that the substitution in the PmrB P95L was a synonymous mutation, or included unknown compensation mutants. It is interesting to note that *pmrCAB* levels were upregulated in strain DC 8253 and contained no mutations of the aforementioned genes, suggesting that other genes seem to be involved in colistin heteroresistance (Figure 1).

Sophie et al identified that the efflux pump inhibitor CCCP could reduce colistin susceptibility in Gram-negative bacteria with diverse molecular mechanisms.[33] The results demonstrated that the reversal effects with a colistin MIC were changed ≥8 fold in all the studied isolates (Table 2). The effect of CCCP on heteroresistant isolates was much greater than that noted on resistant

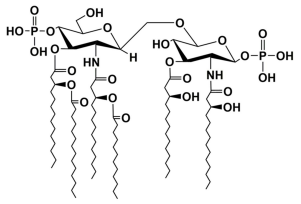
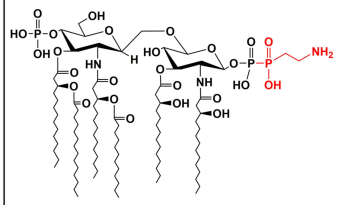
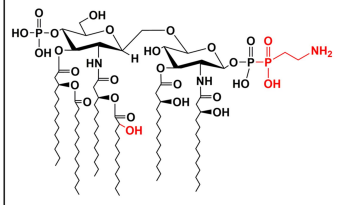
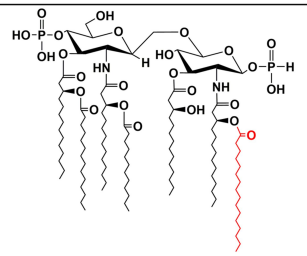
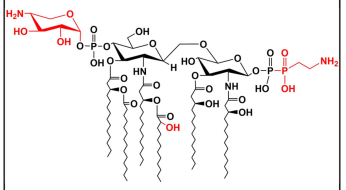
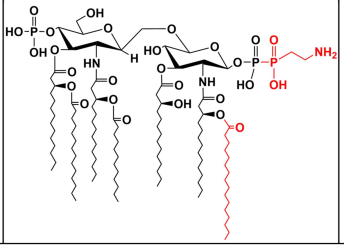
m/z	Structure	Modification
1796		• WT lipid A: the hexa-acyl diphosphoryl lipid A
1919*		• Phosphorylethanolamine (-PEtn) of C-1 phosphate group
1935*		• Phosphorylethanolamine (-PEtn) of C-1 phosphate group • Hydroxylation (-OH) of the C'-2 acy-oxo-acyl chain
2035		• Palmitoylation (-C_{16}) of the C-2 acyl-oxo-acyl chain
2066*		• Phosphorylethanolamine (-PEtn) of C-1 phosphate group • Hydroxylation (-OH) of the C'-2 acy-oxo-acyl chain • Glycosylation (-L-Ara4N) of C-4' phosphate group
2158*		• Palmitoylation (-C_{16}) of the C-2 acyl-oxo-acyl chain • Phosphorylethanolamine (-PEtn) of C-1 phosphate group

Figure 5 Lipid A structures with corresponding *m/z* values found in clinical isolates. Lipid A *m/z* values and molecular structures found in mass spectra of the *E. coli* clinical isolates are shown, with descriptions of the modifications responsible for the observed mass shifts. The asterisks denote ions associated with colistin resistance. The modified moieties are highlighted in red.

isolates, speculating that the inhibitory role of CCCP was more critical for colistin heteroresistance. Furthermore, transcriptomic analysis of regulatory genes was conducted. In the colistin-resistance group, the association of colistin and CCCP inhibited the transcription of the *mcr-1* gene (Figure 2), as previously reported.[26] However, in the colistin heteroresistance group, the higher inhibitory activity with regard to the expression of *pmrC* ranged from 5- to 1000-fold (Figure 3). Interestingly, a certain correlation was noted between the decreased MIC and the altered transcription of the genes. However, it remains unknown how CCCP contributed to the reversal of the susceptibility of colistin. This may have been caused either by inhibition of efflux pumps[19,48–50] or by an unknown action of CCCP,[51] as previously described. Thorough studies are still warranted on the underlying inhibition of efflux pumps in order to fully elucidate the complex mechanisms of colistin resistance and heteroresistance.

It has been reported that the modification of lipid A, such as the addition of L-Ara4N, PEtn and GalN, is associated with colistin resistance.[52,53] In *E. coli*, the addition of PEtn and/or L-Ara4N was associated with chromosome-encoded resistance mechanisms, such as mutations in the PmrAB or PhoPQ TCS.[35] In addition, PEtn can also occur in strains with plasmid-borne *mcr* genes.[54] However, until now, a limited number of studies have reported that the lipid A profile is associated with colistin heteroresistance, notably in *E. coli*. In the present study, all strains were subjected to MALDI-TOF analysis of extracted lipid A to characterize modifications (Figure 4). The results indicated that two colistin-resistant isolates displayed the PEtn peaks (*m*/*z* 1919). The heteroresistant isolate DC 8471 has also demonstrated the PEtn peaks at the *m*/*z* of 1919. The isolates DC 8243 and DC 8253, shared the same MS profile with the PEtn peaks at *m*/*z* of 1919 and 2158, respectively. It is interesting to note that the isolate DC 8446 exhibited PEtn (*m*/*z* 1919) and L-Ara4N (*m*/*z* 2066) peaks, whereas it did not contain non-synonymous mutations of the aforementioned genes. Additional studies regarding the mechanisms of colistin heteroresistance should be performed.

Conclusion

In conclusion, the present study provided the first report for colistin-resistance and heteroresistance in *E. coli*. Colistin exhibited a strong bacteriostatic activity against the tested isolates. The presence of the *mcr-1* gene was the main mechanism of colistin resistance, while the PmrAB TCS mainly contributed to heteroresistance against colistin. The present study further suggested that the action of CCCP was particularly important for colistin heteroresistance. Nevertheless, it remains unclear how relevant this phenotype is with reduced treatment efficacy and a poor clinical outcome. Additional studies are required in the future to fully understand the extent of colistin heteroresistance.

Ethical Statement

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

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed on the journal to which the article will be submitted; gave final approval of the manuscript version to be published; and agreed to be accountable for all aspects of the work. Jianming Cao and Tieli Zhou are joint corresponding authors.

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Disclosure

The authors report no conflicts of interest for this work.

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