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ORIGINAL RESEARCH

Colistin Resistance Mechanisms and Molecular Epidemiology of *Enterobacter cloacae* Complex Isolated from a Tertiary Hospital in Shandong, China

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Background: *Enterobacter cloacae* complex (ECC), which includes major nosocomial pathogens, causes urinary, respiratory, and bloodstream infections in humans, for which colistin is one of the last-line drugs.

Objective: This study aimed to analyse the epidemiology and resistance mechanisms of colistin-resistant *Enterobacter cloacae* complex (ECC) strains isolated from Shandong, China.

Methods: Two hundred non-repetitive ECC strains were collected from a tertiary hospital in Shandong Province, China, from June 2020 to June 2022. Whole-genome sequencing and bioinformatics analyses were performed to understand the molecular epidemiology of the colistin-resistant ECC strains. The nucleotide sequences of heat shock protein (hsp60) were analyzed by using BLAST search to classify ECC. The gene expression levels of *ramA, soxS, acrA, acrB, phoP*, and *phoQ* were assessed using RT-qPCR. MALDI-TOF MS was used to analyse the modification of lipid A.

Results: Twenty-three colistin-resistant strains were detected among the 200 ECC clinical strains (11.5%). The *hsp60* cluster analysis revealed that 20 of the 23 ECC strains belonged to heterogeneous resistance clusters. Variants of mgrB, phoPQ, and pmrAB, particularly phoQ and pmrB, were detected in the 23 ECC strains. The *soxS* and *acrA* genes were significantly overexpressed in all 23 colistin-resistant ECC strains ($P < 0.05$). Additionally, all 23 ECC strains contained modified lipid A related to colistin resistance, which showed five ion peaks at m/z 1876, 1920, 1955, 2114, and 2158. Among the 23 ECC strains, 6 strains possessed a phosphoethanolamine (pETN) moiety, 16 strains possessed a 4-amino-4-deoxy-L-arabinose (−L-Ara4N) moiety, and one strain had both pETN and −L-Ara4N moieties.

Conclusion: This study suggests that diverse colistin resistance existed in ECC, including unknown resistance mechanisms, exist in ECC. Mechanistic investigations of colistin resistance are warranted to optimise colistin use in clinical settings and minimise the emergence of resistance.

Keywords: *Enterobacter cloacae* complex, ECC, epidemiology, colistin, resistance mechanism

Introduction

The *Enterobacter cloacae* complex (ECC) belongs to the Enterobacteriaceae family, which is widely present in nature and can cause skin, wound, urinary system, and soft tissues.[1] Recent studies have shown that species such as *E. asburiae, E. cancerogenus, E. cloacae, E.* chen*gduensis, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. roggenkampii*, and *E. soli* have been assigned to the ECC.[2–5] Furthermore, whole-genome sequencing (WGS) for bacterial species identification has helped in the classification of bacterial species. Previous reports have described the emergence of many multidrug-resistant (MDR) ECC strains.[6,7] Colistin is considered a last-line antimicrobial for the

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treatment of MDR Gram-negative bacteria.[8] Recently, the appearance of high-risk colistin-resistant clones of Enterobacter hormaechei, which possess type VI secretion system (T6SS), adhesins, and virulence genes, has further complicated the selection of treatments.[9] These characteristics not only enhance the pathogen's virulence and competitive edge but also render the therapeutic strategies for these infections more complex and challenging.

Colistin is a cationic lipopeptide that binds to lipid A and induces outer membrane permeabilisation and inner membrane disruption, leading to cell lysis. Consequently, lipid A is mainly modified by the addition of positively charged moieties, phosphoethanolamine (pETN) and/or 4-amino-4-deoxy-L-arabinose (−L-Ara4N), which are regulated by the two-component systems (TCSs) PmrAB and/or PhoPQ in response to environmental signals, such as cationic antimicrobial peptides (CAMPs), low magnesium levels, or acidic pH. According to previous reports, the activation of two-component regulatory systems, PhoPQ and PmrAB, which upregulate the expression of *eptA* and *arn* operons, as well as mutations in *phoPQ, pmrAB*, and *mgrB*, can lead to colistin resistance.[10,11] In addition, the plasmid-mediated colistin resistance gene *mcr*,[12] the small protein gene *ecr*,[13] and the efflux pump[14] have been reported to confer colistin resistance.

However, a study showed that two-component regulatory system PhoPQ may be associated with colistin resistance in *E. cloacae*, whereas PmrAB may not be associated with colistin resistance.[15] Additionally, *phoPQ* and *pmrAB* were not overexpressed in colistin-resistant *E. cloacae* isolates, in contrast to that in other Gram-negative bacteria such as *Klebsiella pneumoniae and Acinetobacter baumannii*.[16,17] A multicentre study in China revealed overexpression of *pmrA* and *pmrB* in polymyxin-resistant *E. cloacae* isolates.[18] All these findings indicate that colistin resistance mechanisms in ECC might be complex and differ from those in other Gram-negative bacteria; therefore, further comprehensive investigation of the colistin resistance mechanism in ECC is needed.

In this study, we aimed to elucidate the mechanisms underlying colistin resistance in ECC strains isolated from Shandong Province. We collected colistin-resistant ECC clinical strains from Shandong Provincial Hospital and performed integrated molecular epidemiological analysis using methods such as phenotype testing, *hsp60* clustering, multilocus sequence typing (MLST), core genome analysis, relative expression detection, and mass spectrometry to enhance our understanding of the development of colistin resistance in ECC strains.

Methods

Bacterial Isolates

Between June 2020 and June 2022, a total of 200 non-repetitive ECC strains were isolated from Shandong Provincial Hospital of China, of which 23 strains were colistin-resistant ECC (11.5%). Among the 23 colistin-resistant ECC strains, 9 were isolated from urine, 6 from sputum, 2 from blood, 2 from secretions, 2 from ascitic fluid, with the remaining 2 isolated from tissue and bile, respectively. All strains were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (BioMérieux, Marcyl'Étoile, France). The strains were stored in 30% glycerol at −80 °C until further use.

Antibiotic Susceptibility Assay

The antibiotic susceptibilities of piperacillin–tazobactam (TZP), aztreonam (ATM), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), imipenem (IMP), amikacin (AK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LEV), trimethoprim–sulfamethoxazole (SXT), and nitrofurantoin (NIT) were analysed using a Vitek-2 Compact system (bioMérieux, France), and the results of the susceptibility assay were interpreted using the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2022). The minimum inhibitory concentrations (MICs) of colistin (CST) and tigecycline (TGC) were analysed by broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB) according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints). Escherichia coli ATCC 25922 was used as a control.

Efflux Inhibitor Assay

To determine whether the efflux pump affected colistin resistance, colistin MICs were determined in the presence of the efflux pump inhibitor, carbonyl cyanide chlorophenylhydrazone (CCCP; Shanghai Yuanye Bio-Technology Co., Ltd).[19] Dimethyl sulfoxide (DMSO; Shanghai Solarbio Bio-Technology Co., Ltd) was used as the solvent for the CCCP stock solutions.

Whole Genome Sequencing and Analysis

DNA was extracted from all colistin-resistant ECC strains by using a commercial genomic DNA extraction kit (Qiagen). Genomic DNA was tested using second-generation high-throughput sequencing technology on the Illumina HiSeq platform (Novogene Co., Ltd., Beijing, China). Illumina sequences were assembled *de novo* using SPAdes version 3.10.[20] Specific identification was performed using JSpeciesWS based on whole-genome sequences (WGSs) (http://jspecies.ribohost.com/jspeciesws/). MLST was analyzed using the MLST database (https://pubmlst.org/ecloacae/), and new alleles and sequence types were submitted to the MLST website for approval. Sequence annotation was conducted using RAST2.0 (https://rast.nmpdr.org/) combined with BLASTP/BLASTN searches against the UniProtKB/Swiss-Prot and RefSeq databases. Annotation of resistance genes was carried out using CARD (http://arpcard.mcmaster.ca).

ECC strains were allocated to their genetic clusters according to partial *hsp60* sequences using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/). The reference strains of each cluster were used as previously reported and were retrieved from GenBank.[21]

Mutations in colistin resistance genes (*pmrA/B, phoP/Q*, and *mgrB*) and deduced protein sequences were analysed using the NCBI website (https://www.ncbi.nlm.nih.gov) and BLAST against the reference genome of *Enterobacter cloacae* ATCC13047 (NCBI GenBank accession no. CP000647).[22] Strain ecl100 represent a colistin-sensitive clinical isolate.

RNA Isolation and Real-Time Fluorescence Quantitative PCR (RT-qPCR)

Total RNA from the 23 ECC strains was extracted using the Bacterial RNA Miniprep Kit (Biomiga, Shanghai, China) according to the manufacturer's instructions. Next, the RNA was reverse-transcribed using the PrimeScript™ RT reagent kit (Takara, Japan) to obtain cDNA.

The transcript levels of *ramA, soxS, acrA, acrB, phoP*, and *phoQ* were normalised to the expression level of the housekeeping gene *rpoB*. The qPCR primers used in this study are listed in Supplementary Table 1. The relative expression levels of these genes in the 23 ECC strains were compared with those in *E. cloacae* ATCC 700323. All tests were conducted in triplicates. Relative fold-changes were calculated using the formula $2^{-\Delta\Delta Ct}$.

Lipid A Characterization by MALDI-TOF MS

Lipid A was extracted referring an acetic acid-based procedure, as previously described,[23–25] and we have made adjustments to it. Below is a description of the adjusted method. Sodium acetate (8.2 mg) was dissolved in deionised water (1 mL) to prepare a 100 mM sodium acetate solution. Subsequently, an appropriate amount of acetic acid was added to the sodium acetate solution and the pH of the solution was set to 4 using a pH meter. Using 1-μL one-time inoculation rings, two rings full of ECC colonies were inoculated in 400 μL of sodium acetate solution (pH 4), and the samples were vortexed for approximately 1 min and then placed in a water bath at 100 °C for 30 min. The samples were removed and vortexed every 5 min for 20s. Subsequently, they were cooled to room temperature. The samples were centrifuged at 8000 × g for 15 min, the supernatant was discarded, 500 μL of 95% ethanol was added to the remaining bacterial sample, and the solution was vortexed for 1 min. Subsequently, the solution was centrifuged at 8000 × g for 3 min, and the supernatant was discarded. After the ethanol was fully volatilised and dried, 100 μL of matrix solvent (chloroform/methanol/water, 12:6:1 [vol/vol]) was added, and the bacterial sample was stirred vigorously using a pipette tip. The mixture was vortexed for 1 min and centrifuged at 5000 × g for 5 min, and the supernatant was used as the lipid extract. Next, 0.75 μL of the lipid extract was spotted on a MALDI-TOF plate followed by spotting of 0.75 μL of norharmane matrix (10 mg/mL in chloroform/methanol/water matrix solvent; Sigma-Aldrich, St. Louis, MO. USA) and the spots were air-dried. The samples were analysed using a ZYbio EXS3000 Autoflex MALDI-TOF mass spectrometer in negative-ion mode.

Pulsed-Field Gel Electrophoresis (PFGE)

The 23 ECC strains were analysed by PFGE to determine their genetic relatedness. In brief, the bacterial genomic DNA embedded in gel plugs was digested with QuickCut *Xba*I (Takara, Shiga, Japan), and DNA fragments were separated using the CHEF Mapper apparatus (Bio-Rad, Hercules, CA, USA) under the following conditions: temperature, 14 °C;

voltage, 6 V/cm; switch angle, 120°; and the pulse time was switched from 2.16 s to 63.8 s for 18 h. PFGE patterns were compared using GelJ software, version 2.0.[26] Pulsotypes were allocated to genetically similar clusters using an 80% cutoff value.[27]

Core Genome MLST (cgMLST)

For our dataset, core genome MLST (cgMLST) analysis was performed using Ridom SeqSphere+ Server (8.0.2 version Ridom, Germany). The resulting set of target genes was used to interpret the clonal relationship displayed in a minimum spanning tree using the 'pairwise ignoring missing values' parameter during distance calculations.

Statistical Analysis

Data were analysed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA). Unpaired Student's *t*-test (two-tailed) was used to compare the significance of the differences in gene expression levels between colistin-resistant clinical ECC and *Enterobacter cloacae* ATCC 700323. Statistical significance was set at $P < 0.05$.

Results

Results of Antimicrobial Susceptibility Testing and Efflux Inhibitor Assay

Of the 200 ECC strains, 23 were resistant to colistin, with MICs ≥ 4 mg/L, and were selected for this study. As shown in Supplementary Table 2, 12 of the 23 colistin-resistant strains were not MDR, and most strains were sensitive to most antibiotics. None of the strains were resistant to carbapenems. When the efflux pump inhibitor CCCP was present, a reduction of at least four-fold in the MICs was considered indicative of efflux.[28] The results of the efflux inhibitor assays are shown in Table 1. In the presence of the efflux pump inhibitor CCCP, the MICs of colistin for all ECC strains decreased significantly to the levels at which the strains became susceptible, suggesting an association between colistin resistance in ECC strains and efflux pumps.

Table 1 Characteristics and Efflux Inhibitor Assay Results of the 23 ECC Strains

Strains	*Enterobacter* spp.	Specimens	MLST	Beta-Lactamase(s)	hsp60 Clusters	Ecr Gene	pETN	—L-Ara4N	Efflux Inhibitor Assay (MIC, mg/L)	
									CST	CST +CCCP
ecl01	*E. bugandensis*	Sputum	ST35	-	IX	-	-	+	512	<0.5
ecl02	*E. roggenkampii*	Sputum	ST2044	-	IV	-	-	+	1024	<0.5
ecl04	*E. bugandensis*	Urine	ST2045	-	IX	-	-	+	8	<0.5
ecl05	*E. roggenkampii*	Urine	ST561	-	IV	-	-	+	256	<0.5
ecl07	*E. cloacae*	Blood	ST524	-	XI	+	-	+	16	<0.5
ecl08	*E. bugandensis*	Secretion	ST2046	-	IX	-	-	+	256	<0.5
ecl09	*E. bugandensis*	Urine	ST2047	-	IX	-	+	+	256	<0.5
ecl11	*E. asburiae*	Tissue	ST2048	-	I	-	-	+	256	<0.5
ecl12	*E. cloacae*	Urine	ST1718	TEM-4, CTX-M-15, OXA-1	XI	+	+	+	64	<0.5
ecl13	*E. roggenkampii*	Sputum	ST272	-	IV	-	-	+	512	<0.5

(*Continued*)

Table 1 (Continued).

Strains	*Enterobacter* spp.	Specimens	MLST	Beta-Lactamase(s)	hsp60 Clusters	Ecr Gene	pETN	−L-Ara4N	Efflux Inhibitor Assay (MIC, mg/L)	
									CST	CST +CCCP
ecl14	*E. ludwigii*	Secretion	ST2049	-	V	-	-	+	32	<0.5
ecl15	*E. chengduensis*	Sputum	ST414	TEM-4, CTX-M-15, OXA-1	IV	-	+	+	1024	<0.5
ecl16	*E. cloacae*	Urine	ST432	TEM-4, CTX-M-15, OXA-1	XI	+	+	-	64	<0.5
ecl17	*E. bugandensis*	Urine	ST2050	-	IX	-	+	-	2048	<0.5
ecl18	*E. kobei*	Urine	ST520	-	II	-	-	+	32	<0.5
ecl19	*E. hormaechei*	Urine	ST151	-	VIII	-	-	+	32	<0.5
ecl20	*E. bugandensis*	Sputum	ST1085	-	IX	-	-	+	2048	<0.5
ecl21	*E. asburiae*	Blood	ST384	-	I	-	-	+	64	<0.5
ecl22	*E. hormaechei*	Ascitic fluid	ST968	CTX-M-15, OXA-1	III	-	-	+	2048	<0.5
ecl24	*E. cloacae*	Ascitic fluid	ST2051	-	XII	+	+	-	32	<0.5
ecl25	*E. kobei*	Urine	ST777	-	II	-	+	+	16	<0.5
ecl26	*E. cloacae*	Bile	ST2052	-	XII	+	-	+	8	<0.5
ecl27	*E. kobei*	Sputum	ST777	-	II	-	+	+	16	<0.5

Notes: + Positive results. - Negative results.
Abbreviations: pETN, phosphoethanolamine; L-Ara4N, 4-amino-4-deoxy-L-arabinose; CST, colistin; CCCP, carbonyl cyanide chlorophenylhydrazone.

Genetic Clusters Based on hsp60 Sequence Analysis

A previous study showed that *hsp60* gene clusters can be used to classify ECC clinical isolates, and colistin heteroresistance appears to be cluster-dependent in ECC strains.[15] Our cluster identification results showed that the 23 strains were divided into nine clusters, and genetic clusters belonging to cluster IX were predominant, accounting for 26.1% (6/23) of the strains, followed by 4, 3, 3, 2, 2, 1, 1, and 1 in clusters IV, II, XI, I, XII, III, V, and VIII, respectively (Table 1).

Genetic Typing, PFGE, and cgMLST Analysis

Eight species were identified based on WGS: *E. bugandensis* (6), *E. cloacae* (5), *E. roggenkampii* (3), *E. kobei* (3), *E. asburiae* (2), *E. hormaechei* (2), *E. chengduensis* (1), and *E. ludwigii* (1) (Table 1). Using MLST, 22 sequence types (STs) were identified, including 9 novel STs (ST2044 – ST2052) (Table 1). In addition to ST777 (n = 2), the remaining STs contained only one strain. MLST diversity was consistent with that observed in the PFGE experiments. Traditionally, PFGE has been considered to be more discriminatory than MLST. Interestingly, ecl12 and ecl16, which belong to similar pulsotypes, exhibited different STs.

PFGE of the 23 ECC strains revealed 21 pulsotypes (Figure 1). Two of these, ecl25 and ecl27, had the same profile, whereas ecl12 and ecl16 had an over 80% value. Additionally, the 19 ECC strains were assigned to different pulsotypes, indicating a distant homology between the strains.

A minimum spanning tree of the 23 ECC strains was constructed based on cgMLST allelic profiles, which resulted in no clustering (Figure 2). This finding indicates that most strains were non-clonal, and resistance to colistin was extensively disseminated in various STs of the ECC strains.

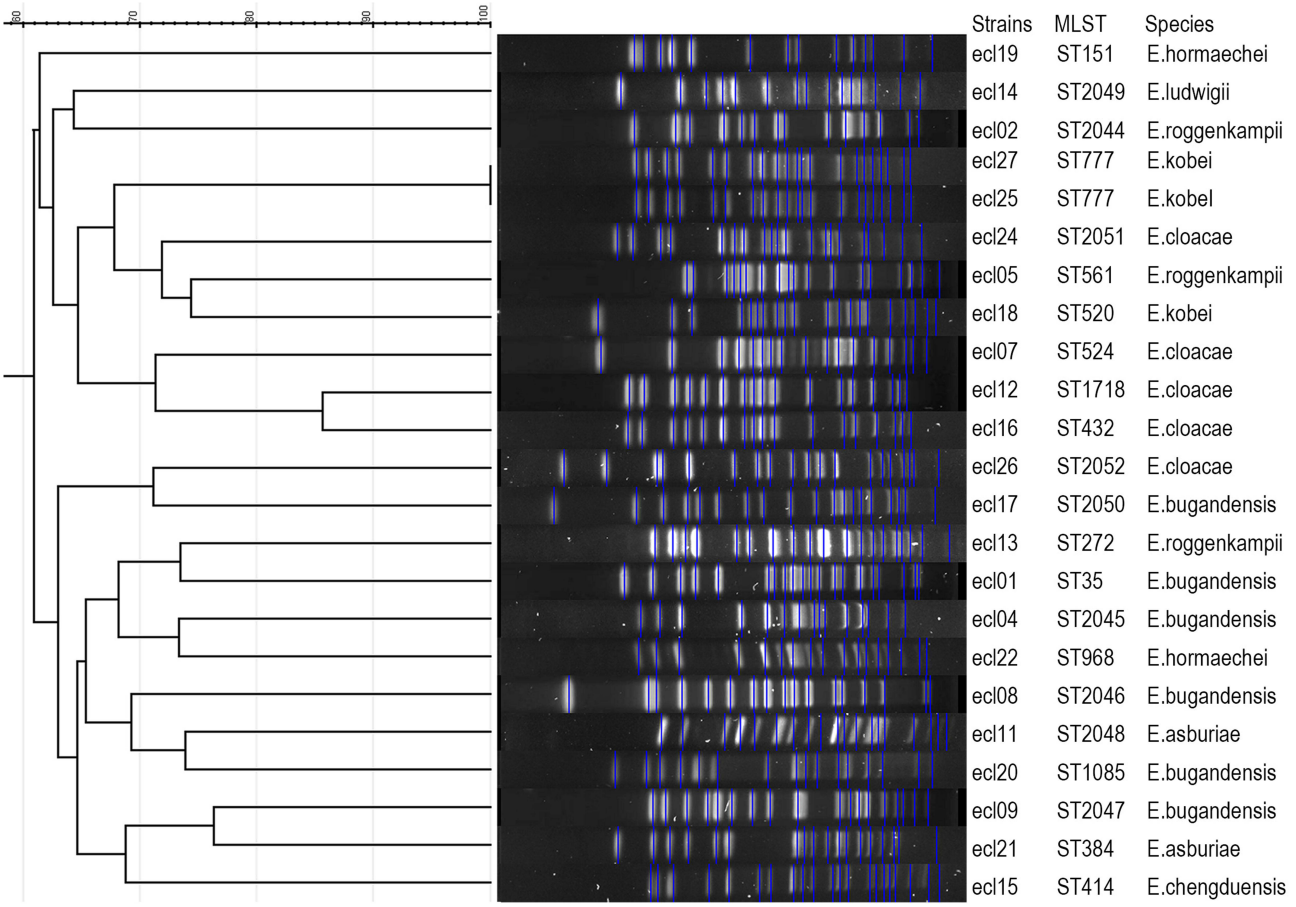

Figure 1 Dendrogram of the pulsed-field gel electrophoresis (PFGE) profiles of 23 clinical colistin-resistant ECC strains. The right panel shows results in the form of strain number, multilocus sequence type and species.
Abbreviations: MLST, multilocus sequence type.

Resistance Mechanism Based on Genetic Analysis

Using CARD, we found that five *E. cloacae* complex strains (ecl07, ecl12, ecl16, ecl24, and ecl26) possessed *ecr* genes, all of which were identified as *E. cloacae* (Table 1). *E. cloacae* ecl12, ecl16, and *E. chengduensis* ecl15 possessed the extended-spectrum β-lactamase genes $bla_{\text{TEM-4}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$, whereas *E. hormaechei* ecl22 only possessed $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ (Table 1). Other *E. cloacae* complex strains did not possess the extended-spectrum β-lactamase gene. Additionally, numerous efflux pump genes (*acrA, acrB, acrD, acrE, acrF, acrR, emrA, emrB, emrD, emrE, emrR, macB, mdfA, mdtA, mdtB, mdtC, mdtG, mdtH, mdtK, mexB, mexQ, msbA, oqxA, oqxB, rosA, rosB, tetC, tolC, vgaC*, and *yojI*) were present in the 23 ECC genomes (Supplementary Table 3).

Amino acid alterations were found in 9, 63, 16, 59, and 5 of the 223 PhoP, 487 PhoQ, 220 PmrA, 347 PmrB, and five MgrB amino acid sites, respectively (Supplementary Table 4). Among these, three PhoP, ten PhoQ, one PmrA, 13 PmrB, and one MgrB sites were identified in only one strain, whereas one PhoP, 29 PhoQ, 4 PmrA, and 32 PmrB amino acid alterations occurred in both colistin-resistant strains and colistin-susceptible strain ecl100 (Supplementary Table 4). Only 5 PhoP and 32 PhoQ amino acid alterations were found in more than five colistin-resistant strains. In addition, 12 PmrA, 28 PmrB, and 1 MgrB amino acid alterations were found in more than five colistin-resistant strains. Overall, 152 amino acid alterations were identified in PhoPQ, PmrAB, and MgrB in 23 ECC strains, 78 of which were found in more than five colistin-resistant strains.

Relative Expression of Efflux Pump Genes and phoPQ

Compared to *Enterobacter cloacae* ATCC 700323, *soxS* and *acrA* were significantly overexpressed in all 23 colistin-resistant ECC strains ($P < 0.05$) (Figure 3). Conversely, *ramA, acrB, phoP*, and *phoQ* were significantly overexpressed in

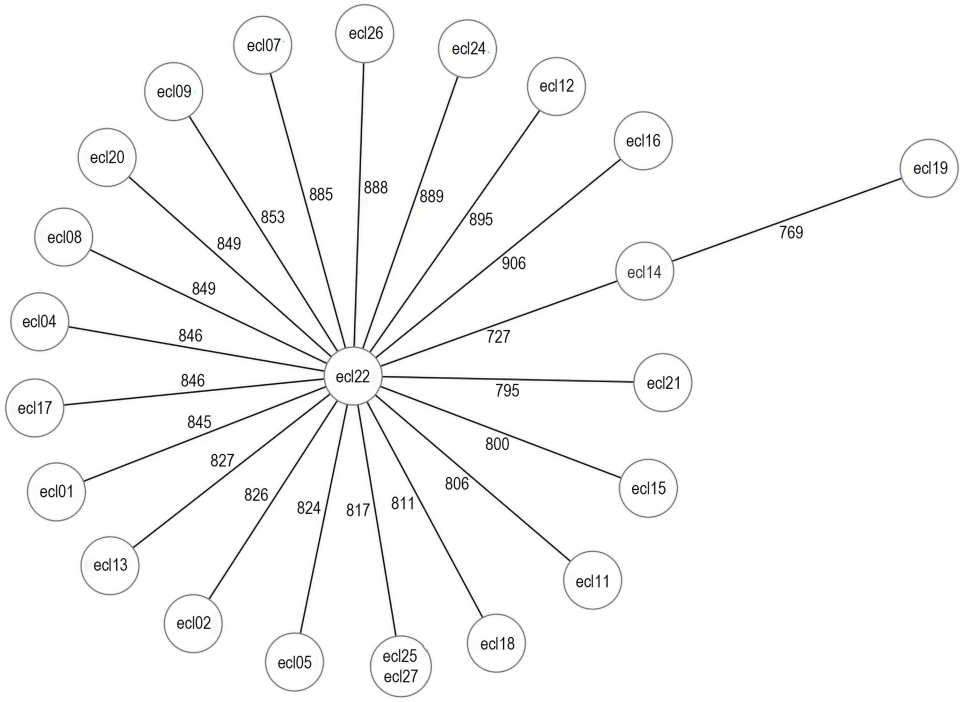

Figure 2 Minimum spanning tree of 23 ECC strains constructed according to core genome multilocus sequence typing (cgMLST). Each circle within the tree represents an allelic profile. The numbers on the connecting lines represent the numbers of target genes with different alleles.

15, 21, 13, and 16 of the 23 colistin-resistant ECC strains, respectively (Figure 3). Overall, nine ECC strains (ecl04, ecl12, ecl13, ecl4, ecl16, ecl17, ecl19, ecl24, and ecl27) significantly overexpressed the six examined genes *ramA, soxA, acrA, acrB, phoP*, and *phoQ*. In addition, five isolates identified as *E. cloacae* ecl07, ecl12, ecl16, ecl24, and ecl26, carrying the *ecr* gene, exhibited overexpression of *phoP* and *phoQ*.

Modifications of Lipid A

The modifications of lipid A in ECC strains are diverse. Among these, the colistin-susceptible clinical strain ecl100 possessed wild-type lipid A with three ion peaks at m/z 1796, 1824, and 2062 (Figure 4). Wild-type lipid A was also present in the 23 colistin-resistant ECC strains, in which the ion peak at m/z 1824 was predominant, which was consistent with a previous report.[19] Interestingly, all 23 ECC strains contained a modified lipid A related to colistin resistance, exhibiting five ion peaks at m/z 1876, 1919, 1955, 2114, and 2158. The *m/z* peaks at 1919 and 2158 were associated with the pETN moiety (*m/z* 123 mass units), whereas *m/z* peaks at 1876, 1955, and 2114 were associated with the −L-Ara4N moiety (*m/z* 131 mass units). Overall, among the 23 colistin-resistant ECC strains, six strains possessed the pETN moiety and 16 strains possessed the −L-Ara4N moiety. Specifically, one strain contained both pETN and L-Ara4N moieties (Table 1).

Discussion

In many cases, colistin has become a last-resort agent for combating infections caused by MDR Gram-negative bacteria.[29] With the rise of MDR ECC,[7] the issue of colistin resistance has garnered significant international attention. Previous studies have shown that the prevalence of colistin resistance in ECC clinical strains was 27.5% in Japan (2017–2018),[30] 11.3–13.3% in the UK and Ireland (2017),[31] and 34.5% in China (2020).[22] In the present study, 11.5% of the ECC clinical strains obtained from Shandong Provincial Hospital in China were colistin-resistant. These findings indicated that colistin-resistant ECC are highly prevalent.

Moreover, *hsp60* clustering has been used to classify ECC clinical strains, and colistin heteroresistance appears to be cluster-dependent in ECC.[15] ECC can be divided into 13 clusters (C-I to C-XIII) based on the sequence of the *hsp60* gene, and clusters III, VI, and VIII were the most frequently recovered from human clinical samples.[21] According to

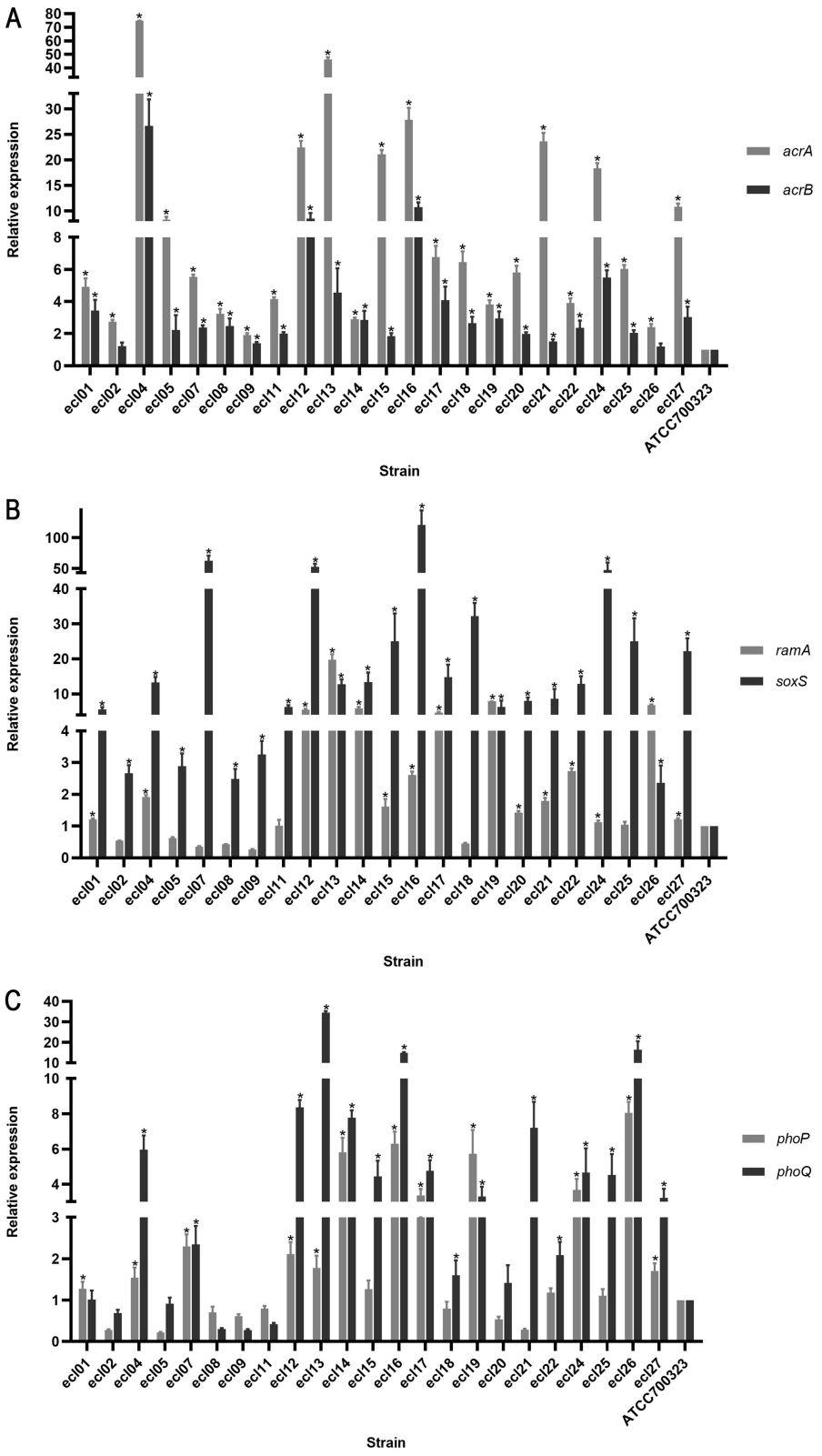

Figure 3 Relative expression of efflux pump protein gene *acrA*/*acrB*, *ramA*/*soxA*, and TCS protein gene *phoP*/*phoQ*.
Notes: (**A**) Expression of *acrA* and *acrB*. (**B**) Expression of *ramA* and *soxS*. (**C**) Expression of *phoP* and *phoQ*. ATCC700323 is the reference strain. *Statistically significant versus ATCC700323.

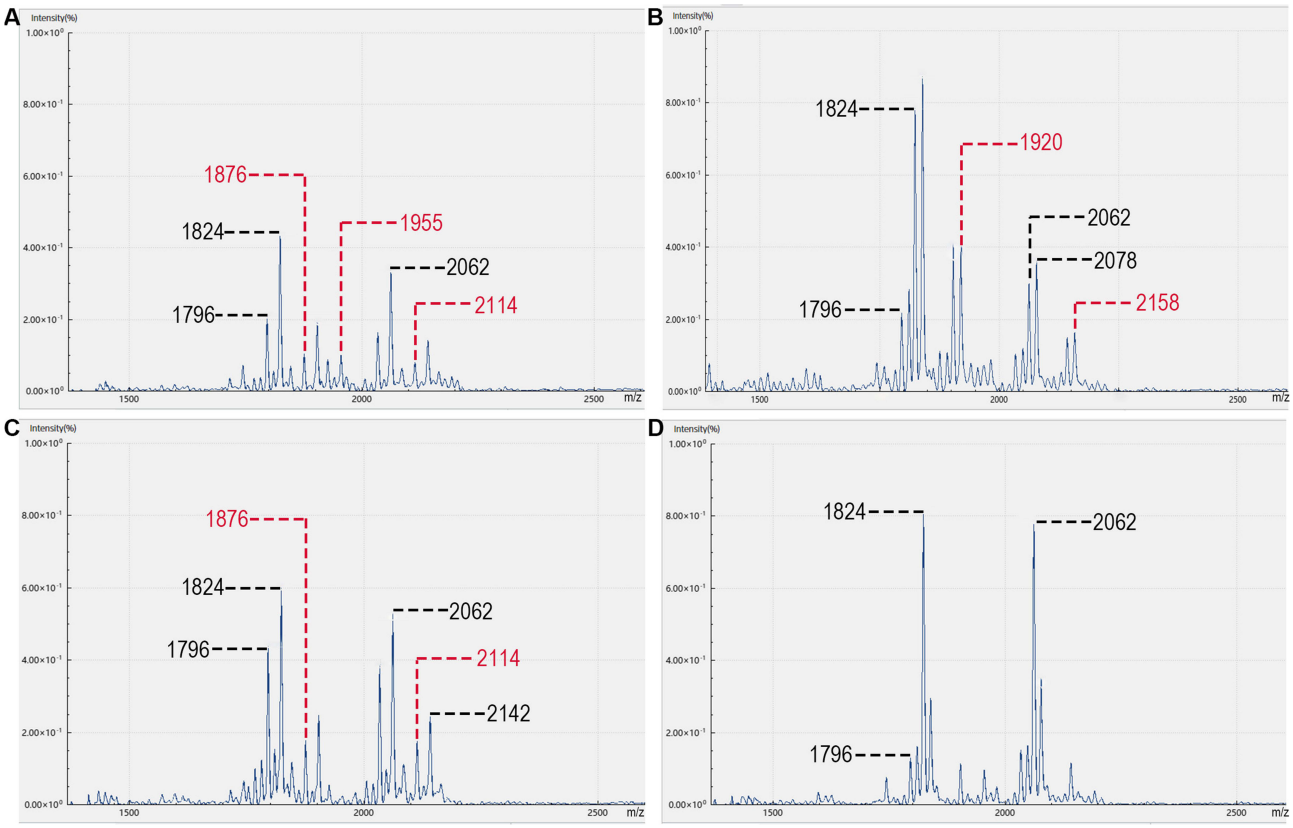

Figure 4 MALDI-TOF mass spectrometry analysis of lipid A.

Notes: Each experiment was independently replicated three times, and one representative data set is reported. Black labeled ion peaks are original peaks or modified peaks not related to colistin resistance. Red labeled ion peaks are the modified peak related to colistin resistance. (**A** and **B**), colistin resistant clinical isolates. (**C**) Positive control strain *Enterobacter cloacae* ATCC13047. (**D**) Colistin sensitive clinical isolate.

previous studies, heteroresistance to colistin depends on the cluster; strains from clusters I, II, IV, VII, IX, X, XI, and XII are usually heteroresistant, whereas those from clusters III, V, VI, VIII, and XIII are categorised as susceptible. Our findings showed that 20 of the 23 ECC strains belonged to heterogeneous resistance clusters, including two, four, four, six, three, and three strains in clusters I, II, IV, IX, XI, and XII, respectively, which could partly explain colistin resistance. Genetic relatedness and phylogenetic analyses showed that only four (ecl12 and ecl16; ecl25 and ecl27) of the 23 ECC strains had a high degree of homology, indicating that colistin-resistant ECC in the tertiary hospital was sporadic.

CCCP is a strong uncoupling agent that acts as an efflux pump inhibitor of SMR-, MFS-, MATE-, and RND-type efflux pumps. Consistent with previous studies,[19] the addition of CCCP to the test medium significantly decreased the MICs for colistin-resistant ECC isolates by at least four-fold, indicating that the acrAB-tolC efflux pump may have evolved to provide active antibiotic efflux in colistin-resistant ECC strains.

AcrA and acrB, which belong to the resistance nodulation division (RND) superfamily, are well-known multidrug efflux pumps that confer resistance to a wide variety of agents.[32] A previous study demonstrated that increased levels of *soxS* and *ramA* transcription activated *acrAB* expression, leading to increased antibiotic resistance in *E. cloacae*.[14] Furthermore, *soxS* simultaneously upregulates expression of *acrA* and *acrB* to induce heterogeneous resistance in *E. cloacae*.[33] In our study, 21 ECC strains showed an increase in the expression levels of *soxS, acrA*, and *acrB*, whereas the remaining two ECC isolates (ecl02 and ecl26) did not show overexpression of *acrB*, although the two strains overexpressed *soxS* and *acrA*. This inconsistency in the expression levels of *soxS, acrA*, and *acrB* in the ecl02 and ecl26 strains may be because *acrA* and *acrB* are not co-regulatory genes, and their expression is usually regulated by multiple levels of different regulatory factors.

Genes involved in colistin resistance, such as those mutated in PmrAB or PhoPQ two-component regulatory system and mgrB inactivation, are usually associated with high levels of colistin resistance.[34] Although some amino acid variations in PhoPQ and PmrAB of colistin-resistant ECC have been reported,[22,35,36] the involvement of these mutations in the colistin-resistant phenotype has not been demonstrated because of a lack of studies on complementation or site-directed mutagenesis. This study also showed variations in both PhoPQ and PmrAB among 23 ECC strains, particularly in PhoQ and PmrB, which were consistent with known mutations as previously reported.[22,37] Nevertheless, although these mutations might contribute to high-level colistin resistance, identification of the specific amino acids involved in colistin resistance is difficult because the mutations are random, and some variations were also detected in colistin-sensitive strains. The high level of variability in PhoPQ and PmrAB in ECC may indicate that their functions were not strictly preserved. Similar to previous studies,[27] we also found that some substitutions were related to the species. For example, the substitutions R276L and P233T in PmrB were found in all E. kobei strains, whereas the substitution I8L in PhoQ was found in all E. roggenkampii strains. Additionally, the substitution I14V in MgrB was uniquely observed in E. ludwigii.[22] All of these findings suggest that chromosome-mediated colistin resistance in ECC might be species-dependent and warrant further investigation.

According to a systematic review of previous reports, mutations in pmrAB, phoPQ, and mgrB affect the phosphorylation of lipid A of lipopolysaccharides through the pmrHFIJKLM (arnBCDATEF or pbgP) cluster, leading to colistin resistance; mgrB inactivation also affect the sensitivity towards colistin.[38] In this study, all 23 colistin-resistant ECC strains were found to modify lipid A via the addition of pETN and/or –L-Ara4N; however, only 17 strains showed overexpression of phoPQ. RamA is known to directly bind and activate the lpxC, lpxL-2, and lpxO genes associated with lipid A biosynthesis, resulting in modifications within the lipid A moiety of the lipopolysaccharide, which leads to reduced susceptibility to colistin.[39] This report may explain the colistin resistance mechanism of strain ecl20 which shows overexpression of *ramA* accompanied by a reduction in the expression levels of *phoPQ*. The regulatory pathways of the remaining five strains (ecl02, ecl05, ecl08, ecl09, and ecl11) that do not overexpress *ramA* and *phoPQ* but harbour modified lipopolysaccharide (LPS) require further investigation.

A previous study showed that colistin heteroresistance in ECC is mediated by a new small transmembrane protein-encoding gene, *ecr*, which was hypothesised to activate the *arnBCADTEF* operon via the PhoPQ system.[13] Interestingly, all of the five colistin-resistant ECC strains possessed *ecr* genes were identified as *E. cloacae*, thus the relationship between *ecr* gene and ECC species need further investigation. In addition, among the five strains, we observed that three strains modified lipid A via the addition of pETN and the remaining two strains modified lipid A via the addition of −L-Ara4N. As a positive control, our findings showed that −L-Ara4N was modified to LPS in *Enterobacter cloacae* ATCC 13047, which was consistent with previous findings that colistin heteroresistance in *Enterobacter cloacae* ATCC 13047 was due to the addition of L-Ara4N to LPS, which was mediated by enzymes associated with the *arnBCADTEF* operon under the control of the PhoPQ two-component system.[40] However, manual extraction of lipids is complicated, so we need to improve and standardise diagnostic kits for the detection of colistin resistance in clinical laboratories.

Although our investigation did not identify mcr gene-carrying ECC strains, a recent study conducted in China has reported concerning prevalence rates of 10.7% for mcr-9 and 4.3% for mcr-10 in ECC, respectively,[41] which may be caused by the limited number of strains we collected. This highlights the need for ongoing vigilance and the implementation of proactive measures to prevent the global spread of mcr-mediated colistin resistance.

Conclusions

In general, our study highlights the multifaceted molecular mechanisms of colistin resistance in ECC, and the main mechanisms of colistin resistance elucidated in this study are overexpression of the efflux pump-associated genes *acrA, acrB, ramA*, and *soxA*, accompanied by lipid A modification via the addition of phosphoethanolamine (pETN) and/or 4-amino-4-deoxy-L-arabinose (−L-Ara4N), which may result from mutations and overexpression of two-component systems (TCSs) PmrAB and PhoPQ. Hence, it is urgent to take measures to control and prevent the prevalence of colistin resistance and further investigate the colistin resistance mechanisms in ECC.

A recent review of polymyxin points out nanotechnology could be a frontier in optimizing antibiotic delivery and performance.[42] Another study has demonstrated a novel strategy for overcoming colistin resistance in bacterial infections

through the co-delivery of curcumin and colistin encapsulated in negatively charged polyethylene glycol-functionalized liposomes.[43] These research efforts are crucial to minimize the development of resistance and prolong the effectiveness of colistin as a last-line treatment.

Abbreviations

AK, amikacin; ATM, aztreonam; CAMHB, cation-adjusted Mueller-Hinton broth; CAMP, cationic antimicrobial peptide; CAZ, ceftazidime; CCCP, carbonyl cyanide chlorophenylhydrazone; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; CRO, ceftriaxone; CST, colistin; DMSO, dimethyl sulfoxide; ECC, *Enterobacter cloacae* complex; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FEP, cefepime; GEN, gentamicin; Hsp60, 60-kDa heat shock protein; IPM, imipenem; −L-Ara4N, 4-amino-4-deoxy-L-arabinose; LEV, levofloxacin; LPS, lipopolysaccharide; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MLST, multilocus sequence type; NIT, nitrofurantoin; pETN, phosphoethanolamine; PFGE, pulsed-field gel electrophoresis; RT-qPCR, real-time fluorescence quantitative; SXT, trimethoprim-sulfamethoxazole; TCS, two-component system; TGC, tigecycline; TOB, tobramycin; TZP, piperacillin-tazobactam; WGS, whole.

Data Sharing Statement

The DNA sequences of the 23 colistin-resistant ECC strains were deposited in the NCBI GenBank database (BioProject PRJAN1085784).

Ethics Approval

All the clinical samples used in this study were part of the routine hospital laboratory procedure, and no personal identifiers were used throughout the study. This research was identified as a non-human participant study, focusing on laboratory analysis of bacteria, which aligns with the regulations of our research institution that exempt it from ethical approval. Furthermore, considering the severe threat posed by colistin-resistant strains to public health, the study of their molecular epidemiology and mechanisms of drug resistance is of significant importance for the development of novel treatment strategies and the control of antimicrobial resistance dissemination. It is regarded as an urgent research that serves the public interest.

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Author Contributions

All authors made a significant contribution to the work, whether in conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas. They took part in drafting, revising, or critically reviewing the article, approved the version to be published, agreed on the journal to which the article has been submitted, and agreed to be accountable for all aspects of this work.

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Disclosure

The authors report no competing interests in this work.

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