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ORIGINAL RESEARCH

Emergence, Molecular Characteristics and Resistance Mechanisms of Colistin-Resistant *Enterobacterales* in Xuzhou, China

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Background: Carbapenem-resistant Enterobacterales (CRE) infections pose a significant global public health threat, with colistin as the last line of defense. Increasing colistin resistance presents a formidable clinical challenge. This study aimed to elucidate the molecular characteristics and resistance mechanisms of clinical colistin-resistant (ColR) CRE strains in Xuzhou, China.

Methods: The broth microdilution method and PCR were performed to detect antibiotic resistance phenotype and resistance genotype. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to determine genetic relatedness. Whole-genome sequencing (WGS) was carried out to characterize plasmids carrying resistance genes. Phylogenetic analysis was conducted by constructing phylogenetic tree based on single nucleotide polymorphism (SNP) and core genome multilocus sequence typing (cgMLST).

Results: In 14 ColR-carbapenem-resistant *Klebsiella pneumoniae* (ColR-CRKP) strains, the inactivation of the *mgrB* gene leads to colistin resistance. The *mcr-1* gene, carried by a plasmid, mediated colistin resistance in 4 ColR-carbapenem-resistant *Escherichia coli* (ColR-CREC) strains. PFGE revealed potential cloning epidemics in both ColR-CRKP and ColR-CREC. WGS of *E. coli 104* demonstrated the distribution of multiple crucial resistance genes on four plasmids. Notably, *mcr-1* was located on the IncI2 plasmid while $bla_{\text{NDM-5}}$ was located on the IncFII plasmid. Phylogenetic trees, based on SNP and cgMLST, illustrate that the clonal epidemic strains, as exemplified by *E. coli* 104, have the potential to spread across regions and species.

Conclusion: This study underscores that mutations in the *mgrB* gene and the presence of *mcr-1* contribute to the development of colistin resistance in CRE. Additionally, it enriches local epidemiological knowledge, facilitating a better understanding and control of the spread of *mcr-1*.

Keywords: colistin, *mgrB*, *mcr-1*, carbapenem-resistant, *Enterobacterales*

Introduction

The widespread prevalence of extended-spectrum β-lactamases (ESBLs) within Enterobacterales has led to the extensive utilization of carbapenems in clinical therapy.[1] Consequently, this selective pressure has facilitated the emergence and spread of carbapenem-resistant Enterobacterales (CRE) through the transfer of plasmid-mediated genes, such as $bla_{\text{NDM-1}}$, bla_{IMP}, bla_{KPC}, and $bla_{\text{OXA-48}}$.[2] Therefore, CRE has emerged as a predominant pathogen in clinical infectious diseases, posing a significant public health challenge.[3] This shift towards carbapenem resistance not only prolongs hospital stays and increases mortality rates but also forces clinicians to rely on polymyxins, particularly colistin, as the last line of defense.[4] Understanding the mechanisms and epidemiology of colistin resistance is therefore crucial to preserving the efficacy of this vital antibiotic and informing strategies to mitigate the spread of antimicrobial resistance.

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Given the increasing prevalence of carbapenem resistance, polymyxin antibiotics, including polymyxin B and colistin, have emerged as a crucial last line of defense against CRE.[4] These cationic compounds exert their antibacterial action by binding to the negatively charged lipid A component of lipopolysaccharide (LPS) in the bacterial cell membrane, thereby disrupting membrane integrity and inducing bacterial death. However, the emergence of resistance to polymyxins further complicates clinical treatment strategies.

One key mechanism of colistin resistance involves the acquisition of the *mcr* gene. The *mcr* gene encodes a phosphoethanolamine transferase that catalyzes the addition of phosphoethanolamine (PEtN) to the lipid A component of lipopolysaccharides (LPS) in the bacterial outer membrane.[5] This modification reduces the net negative charge of the bacterial cell surface, consequently decreasing the binding affinity of the cationic peptide colistin and conferring resistance.

Intrinsic (chromosomally mediated) resistance to colistin primarily stems from modifications to lipid A that alter its charge. Key modifications include the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and PEtN to lipid A.[6] These additions mask the negative charge of the cell envelope, thereby reducing the electrostatic interaction with polymyxins like colistin. Additionally, chromosomally encoded mutations, particularly in genes governing two-component regulatory systems (TCSs) such as PhoP-PhoQ and PmrA-PmrB, can lead to constitutive overexpression of LPS modification genes. These TCSs normally sense environmental cues (eg, low Mg^{2+} or Fe^{3+}) to induce adaptive LPS changes; however, gain-of-function mutations disrupt this regulation, resulting in persistent LPS alterations and resistance. Notably, inactivation of the *mgrB* gene, a key negative regulator of the PhoP-PhoQ system, represents a common mutational pathway that derepresses the system and upregulates modification genes, thereby promoting colistin resistance.[7]

In contrast to these chromosomally mediated mechanisms, the plasmid-borne *mcr* gene, first identified in China in 2015, enables horizontally acquired resistance. It has since been reported globally, predominantly in *Escherichia coli*.[8] To date, ten distinct variants of the *mcr* gene family (*mcr-1* to *mcr-10*) have been identified worldwide. The horizontal transfer potential of these genes between diverse bacterial species represents a significant public health threat.[9]

Against this backdrop of global antimicrobial resistance challenges, Xuzhou, as a major medical hub in eastern China, is characterized by its abundant medical resources and high patient turnover, making it a high-risk area for the spread of CRE. Moreover, the extensive use of antimicrobial agents, especially during the COVID-19 pandemic, may have further exacerbated the drug resistance of CRE. Therefore, an in-depth investigation into the epidemiology and resistance mechanisms of CRE in Xuzhou is crucial for developing targeted public health strategies and antimicrobial stewardship programs. This study focuses on colistin-resistant CRE strains in Xuzhou, aiming to provide scientific evidence for regional prevention and control through epidemiological surveys and resistance mechanism analyses to address this increasingly severe public health challenge.

Materials and Methods

Bacterial Isolates

Between May 2016 and June 2022, 18 non-duplicated clinical isolates of colistin-resistant CRE were collected from inpatients at the Affiliated Hospital of Xuzhou Medical University. These isolates were specifically selected to investigate the prevalence and characteristics of colistin resistance among CRE strains in this clinical setting. Concurrently, detailed clinical information was gathered for each patient, including demographics (age and sex), underlying medical conditions, history of invasive procedures, duration of hospital stay, antimicrobial usage, and clinical outcomes. This comprehensive data collection provided a robust background for the study, facilitating a deeper understanding of the epidemiology and clinical impact of colistin-resistant strains.

For quality control, the colistin-sensitive *K. pneumoniae* wild strain (ATCC700603) was used, which has an intact and functionally normal two-component regulatory systems, such as PhoP-PhoQ and PmrA-B, serving as a reliable reference for susceptibility. Additionally, *Salmonella enterica* H9812 was employed as a reference strain for PFGE analysis due to its well-characterized genome size, which is crucial for accurate and reproducible results. The initial detection of colistin resistance was performed using the broth microdilution method according to CLSI guidelines (2024 edition).

Testing of Antimicrobial Susceptibility

Antimicrobial susceptibility was assessed using the VITEK-2 compact system (bio-Mérieux, Marcy-l'Étoile, France). Minimum Inhibitory Concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M100, 2024).

Genotyping of Isolates

Whole-cell DNA from clinical strains was embedded in agarose gel plugs and subjected to pulsed-field gel electrophoresis (PFGE) using XbaI-digested genomic DNA. The PFGE conditions were as follows: pulse times of 6–36 seconds, pulse angle of 120°, voltage of 6 V/cm, and a running time of 20 hours. The Lambda Ladder PFG Marker (H9812) was used as the molecular weight marker. Clonal relationships were determined based on PFGE profiles, following the criteria proposed by report.[10] Strains with PFGE profiles showing ≥85% similarity were considered clonally related, while those with <85% similarity were considered distinct clones.

Multilocus sequence typing (MLST) was also performed for these isolates using whole-genome sequencing (WGS) data, as previously described.[11] Briefly, sequences of seven housekeeping genes (eg, *gyrA*, *rpoB*, *recA*, *mdh*, *pgi*, *phoE*, *fumC*) were extracted from the WGS data and compared to the MLST database to determine the sequence type (ST) of each isolate. This approach leverages the comprehensive coverage of WGS to provide accurate and detailed MLST results.

The genetic relatedness among the isolates was assessed by combining PFGE and MLST analyses. This dual approach provided a comprehensive understanding of the genetic diversity and clonal relationships among the isolates, and helped identify any potential clonal outbreaks.

Molecular Characterization and Related Genes Analysis

PCR was performed to identify carbapenem-resistant genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) and colistin-resistant related genes (*mcr-1*, *mgrB*), based on a previous study[12] (Table 1). The cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Positive PCR products were sequenced and compared to GenBank using BLAST (www.ncbi.nlm.nih.gov/GenBank).

The expression of *pmrA*, *pmrB*, *pmrC*, *pmrD*, *pmrE*, *pmrK*, *phoQ*, *phoP*, and *mgrB* genes was analyzed using qRT-PCR. The selected genes *(pmrA, pmrB, pmrC, pmrD, pmrE, pmrK, phoP, phoQ, and mgrB*) were chosen because they play critical roles in lipopolysaccharide (LPS) modification and regulation of cell envelope stress responses, which are essential for colistin resistance. Specifically, the *mgrB* gene is involved in the regulation of the PhoP-PhoQ two-component system, which affects LPS modification and colistin binding affinity. The qRT-PCR assays were conducted using the $^{\Delta\Delta}$Ct method for relative quantification, with the *rpsL* gene serving as the internal reference. The *rpsL* gene was chosen due to its stable expression across all isolates tested, which allows for accurate correction of sample-to-sample variations. The qRT-PCR cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each assay was performed in triplicate to ensure data reliability.[13]

The *rpsL* gene was chosen as the internal reference due to its stable expression across all isolates tested. *rpsL* encodes ribosomal protein S12 and has been shown to be highly stable in various bacterial species, making it suitable for normalizing gene expression levels. This stability allows for accurate correction of sample-to-sample variations, thereby enhancing the reliability of experimental results.[14]

mgrB Sequencing: The *mgrB* gene was sequenced using Sanger sequencing to identify specific mutations associated with colistin resistance. The sequencing results were analyzed using the CLC Main Workbench software.

Whole Genome Sequence Analysis

Whole-genome sequencing (WGS) was performed on selected strains to identify the presence of resistance genes and to understand the genetic context of colistin resistance. The coexistence of multiple resistance genes, such as *mcr-1* and bla_{NDM-5}, was observed in some strains, which may contribute to the observed resistance levels.

Table 1 Primers Used in This Study

PCR(Gene)	Primer	Sequence (5'-3')	Amplicon Size (bp)
bla_{KPC}	F	AACAAGGAATATCGTTGATG	916
	R	AGATGATTTTCAGAGCCCTTA	
bla_{NDM}	F	AGCACACTTCCTATCTCGAC	512
	R	GGCGTAGTGCTCAGTGTC	
bla_{IMP}	F	GGYGTTTWTGTTCATACWTCKTTYGA	404
	R	GGYARCCAAACCACTASGTTATCT	
bla_{VIM}	F	AGTGGTGAGTATCCGACAG	261
	R	ATGAAAGTGCGTGGAGAC	
bla_{OXA-48}	F	ATGCGTGTATTAGCCTTATCGG	438
	R	GCGTGGTTAAGGATGAACAC	
mcr-1	F	CGGTCAGTCCGTTTGTTC	309
	R	CTTGGTCGGTCTGTAGGG	
mgrB	F	CTTAGCGCTCTGCCACCAG	253
	R	ATTATTTGTATGATCCTTGCCGT	
qRT-PCR			
pmrA	F	TACGCCGAAAGAGTATGCCC	170
	R	GGATCCGCGATTTGCCAATC	
pmrB	F	TGC CAG CTG ATA AGC GTC TT	95
	R	TTC TGG TTG TTG TGC CCT TC	
pmrC	F	GCG TGA TGA ATA TCC TCA CCA	116
	R	CAC GCC AAA GTT CCA GAT GA	
pmrD	F	GAT CGC AGA GAT TGA AGC CT	120
	R	GCG TTG CGG ATC TTC AAA GT	
pmrE	F	GCA TAC CGT AAT GCC GAC TA	119
	R	GGG TTG ATC TCT GTG ACA TC	
pmrK	F	AGT ATC GGT CAG TGG CTG TT	123
	R	CCG CTT ATC ACG AAA GAT CC	
phoP	F	ATTGAAGAGGTTGCCGCCCGC	136
	R	GCTTGATCGGCTGGTCATTCACC	
phoQ	F	ATATGCTGGCGAGATGGGAAAACGG	138
	R	CCAGCCAGGGAACATCACGCT	
mgrB	F	CCTGTTGCTGTGGACTCAGA	73
	R	AGTGCAAATGCCGCTGAAAA	
rpsL_F	F	CCGTGGCGGTCGTGTTAAAGA	109
	R	GCCGTACTTGGAGCGAGCCTG	

We selected *Escherichia coli* strain 104 as a representative of the same sequence type (ST) for detailed genetic analysis. The genome of this strain was sequenced using both PacBio *RS* Sequel II and Illumina HiSeq 4000 platforms at BGI Technology Service Co., Ltd. (Shenzhen, China) to elucidate the genetic environment surrounding the *mcr-1* gene. The combined approach leverages the long-read capability of PacBio (average read length: 10,000 bp) and the high accuracy of Illumina (coverage: >100x), ensuring comprehensive and accurate genome assembly. The whole genome was submitted to GenBank (GenBank Accession Numbers: CP141581-CP141585).

BLAST was utilized to compare plasmid sequences, The genes associated with drug resistance were predicted using the ResFinder 4.1 database.

Plasmid genome circles were mapped using the CGView server (CGView - Overview), with input files in XML format. Plasmid replicon prediction, single nucleotide polymorphism (SNP)-based phylogenetic trees, and core genome multilocus sequence typing (cgMLST) were executed using BacWGSTdb (BacWGSTdb). The results were enhanced by

TVBOT (TVBOT), which provides interactive visualization of phylogenetic trees. The specific parameters used for SNP analysis in BacWGSTdb included a minimum coverage of 10x and a core genome threshold of 90%. The phylogenetic trees were visualized using TVBOT's drag-and-drop interface, allowing real-time updates and rendering of changes.

Statistical Analysis

Statistical analyses were performed using STATA version 17.0 (StataCorp LLC, College Station, TX, USA). For gene expression analysis by qRT-PCR, the $^{\Delta\Delta}$Ct method was employed, and p-values were calculated using the Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Baseline Information

A total of 18 non-duplicated clinical isolates of colistin-resistant CRE were collected from 18 adult patients admitted to the Affiliated Hospital of Xuzhou Medical University during the study period. Each isolate was derived from a unique patient, ensuring that the sample set represented a diverse range of clinical cases. These isolates included 14 colistin-resistant carbapenem-resistant *Klebsiella pneumoniae* (ColR-CRKP) strains and 4 colistin-resistant carbapenem-resistant *Escherichia coli* (ColR-CREC) strains.

The age of the patients ranged from 18 to 82 years. All patients had underlying infectious or chronic diseases, including sepsis, urinary tract infections, chronic cardiovascular diseases, and cerebrovascular diseases. They had undergone invasive procedures such as surgery, urinary catheterization, tracheal cannula insertion, tracheotomy, and central venous catheterization. The majority of the patients (11/18, 61.1%) had been admitted to the intensive care unit (ICU) and had received advanced antimicrobial agents targeting gram-negative bacteria. These agents included third-generation cephalosporins and enzyme inhibitors (17/18, 94.4%) and carbapenems (10/18, 55.6%). Notably, none of the patients had a history of polymyxin use before the first positive culture for CRE.

Among the 18 patients with colistin-resistant CRE infections, 7 (38.9%) succumbed to the infection, while 11 (61.1%) showed clinical improvement (Table 2).

Antimicrobial Susceptibility

All 18 colistin-resistant CRE strains exhibited multidrug resistance (Table 3). These strains were resistant to numerous antibiotics but showed selective sensitivity to amikacin, sulfamethoxazole, doxycycline, and tigecycline. Specifically, all 18 strains demonstrated high-level colistin resistance, with MIC values ranging from 4 to 16 mg/L.

Among them, the 4 ColR-CREC strains harboring the *mcr-1* gene exhibited low-level resistance (MIC= 4 mg/L). The 14 ColR-CRKP strains with *mgrB* mutations had higher MIC values (≥8 mg/L).

Genotyping and Phylogenetic Analysis

Multilocus Sequence Typing (MLST)

MLST analysis revealed that ST11 (93.0%, 13/14) was the predominant sequence type among the 14 ColR-CRKP strains, indicating a high degree of clonality within this group.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis further subdivided the 14 ColR-CRKP isolates into 9 distinct clusters (A-I), with clusters A and F being the most prevalent. This suggests the presence of multiple clonal lineages within the ST11 group. The four ColR-CREC strains were divided into two PFGE types: a type A clone (ST167) and a sporadic type B (ST405).

SNP-Based Phylogenetic Analysis

SNP-based phylogenetic analysis was performed using the CSI Phylogeny 1.4 tool with the following parameters: minimum coverage of 10x, minimum variant frequency of 0.75, and a core genome threshold of 90%. The resulting phylogenetic tree was constructed using the TVBOT online tool, chosen for its user-friendly interface and ability to

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Table 2 Demographics and Clinical Characteristics of Patients with Colistin-Resistant *K. Pneumoniae* and *E. Coli* Infections

Strain ID	Sex	Age (Years)	Unit	Comorbidity	Invasive Operation	Dates Of Hospital Stay	History of Antibacterial Use	Date Isolate Identified	Specimen	Antibiotic Treatment	Outcome
KP01	M	78	ICU	Fever/sepsis	Urinary catheter, Central venous catheter	17/04/2018-21/05/2018	CRO+MEM+SCF	07/05/2018	Sputum	SCF+TGC	Died
KP02	F	53	NES	Urinary tract infection/fever	Urinary catheter	11/05/2018-27/06/2018	CAZ+BPM+SCF	25/06/2018	Urine	SCF	Improvement
KP03	M	66	NICU	Urinary tract infection	Urinary catheter	21/06/2018-12/07/2018	SCF	28/06/2018	Urine	IMP	Improvement
KP04	M	26	ICU	Fever/sepsis	Urinary catheter, Central venous catheter, tracheotomy	05/01/2019-17/02/2019	SCF	24/01/2019	Blood	SCF+IMP	Died
KP05	M	33	ICU	Pulmonary infection/fever	Urinary catheter	25/03/2019-05/05/2019	SCF+MEM	15/04/2019	Sputum	SCF+MEM	Died
KP06	M	56	NICU	Fever/infection	Urinary catheter, Central venous catheter	27/04/2019-08/05/2019	CRO+MEM+SCF	04/05/2019	Sputum	MEM+SCF+TGC	Improvement
KP07	F	66	URS	Ureteral calculi, Hydronephrosis, Urinary tract infection	Urinary catheter, Central venous catheter	28/04/2019-15/05/2019	CAZ+SCF+MEM	05/05/2019	Urine	SCF+MEM	Improvement
KP08	M	76	NES	Cerebral, Pulmonary infection, Hypertension	Urinary catheter, Central venous catheter	23/05/2019-04/07/2019	SCF+TGC	28/05/2019	Sputum	SCF+TGC	Improvement
KP09	M	62	NES	Meningioma, Pulmonary infection	Urinary catheter, Central venous catheter	15/05/2019-14/06/2019	SCF+TGC	25/05/2019	Sputum	SCF+TGC	Died
KP10	M	18	ICU	Intracranial infection, Pulmonary infection, Cerebral vascular malformation	Urinary catheter, Central venous catheter, tracheotomy	17/05/2019-10/07/2019	SCF+TGC+MEM +PB	28/06/2019	Cerebrospinal fluid	SCF+TGC+MEM +PB	Died
KP11	F	65	NICU	Pulmonary infection	Urinary catheter	05/10/202-26/10/2021	SCF+TGC	12/10/2021	Sputum	SCF+TGC	Died
KP12	F	82	NICU	Pulmonary infection/fever	Urinary catheter, Central venous catheter	12/05/2022-10/06/2022	SCF+TGC	18/05/2022	Sputum	SCF+TGC	Improvement
KP13	F	64	NICU	Fever/infection	Urinary catheter, Central venous catheter, tracheotomy	21/05/2022-25/07/2022	SCF+MEM+TGC	08/06/2022	Sputum	SCF+MEM+TGC +PB+CZA	Improvement
KP14	M	69	RICU	Fever/sepsis	Urinary catheter, Central venous catheter	29/05/2022-19/07/2022	SCF+TGC+BPM	11/06/2022	Sputum	SCF+TGC	Died
E. coli 101	F	55	URS	Bladder tumor	Unknown	27/05/2019-10/06/2019	Unknown	31/05/2019	Urine	Unknown	Improvement
E. coli 102	M	64	DH	Leukemia	Unknown	27/05/2018-10/06/2018	CAZ+LEV+SCF	31/05/2020	Sputum	LEV+SCF	Improvement
E. coli 103	M	26	DH	Leukemia	Unknown	17/07/2017-10/08/2017	SCF+IMP	31/07/2020	Sputum	SCF+IMP	Improvement
E. coli 104	F	64	URS	Kidney stone	Unknown	07/11/2016-18/11/2016	CAZ+IMP	10/01/2022	Urine	CAZ+IMP	Improvement

Abbreviations: M, male; F, female; KP, K. *pneumoniae*; NES, Department of neurosurgery; URS, Department of urinary surgery; ICU, Intensive Care Unit; NICU, Neurology Intensive Care Unit; RICU, Respiratory Intensive Care Unit; DH, Department of Hematology; CRO, Ceftriaxone; CAZ, Ceftazidime; MEM, Meropenem; SCF, Cefoperazone/sulbactam; TGC, Tigecycline; PB, Polymyxin; BPM, Biapenem; CZA, Ceftazidime/avibactam; LEV, Levofloxacin.

Table 3 Antimicrobial Resistance Profiles (MIC, mg/L) of 18 Colistin-Resistant CRE Strains

No.	DC	A/C	AK	CIP	IMP	MEM	AM	TZP	SXT	SCF	ATM	CZ	CPM	CRO	GM	LEV	TOB	TGC	Colistin/Polymyxin B
KP01	>=16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	2	>=16
KP02	>=16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	1	>=16
KP03	8	>=32	8	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=05	>=16
KP04	>=16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=05	>=16
KP05	4	>=32	4	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	8	1	>=16
KP06	>=16	>=32	2	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=05	>=16
KP07	>=16	>=32	<=2	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=05	>=16
KP08	>=16	>=32	<=2	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	8	1	>=16
KP09	4	>=32	4	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	8	2	>=16
KP10	4	>=32	>=64	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	1	8
KP11	4	>=32	>=64	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	>=16
KP12	>=16	>=32	8	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	4	<=0.5	>=16
KP13	8	>=32	>=64	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	<=1	<=0.5	8
KP14	4	>=32	>=64	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	>=16
E. coli 101	16	>=32	4	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	4
E. coli 102	16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	4
E. coli 103	16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	>=320	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	4
E. coli 104	16	>=32	>=64	>=4	>=16	>=16	>=128	>=128	<=20	>=64	>=64	>=64	>=64	>=64	>=16	>=8	>=16	<=0.5	4

Abbreviations: DC, Doxycycline; A/C, Amoxicillin clavulanic acid; AK, Amikacin; CIP, Ciprofloxacin; IMP, Imipenem; MEM, Meropenem; AM, Ampicillin; TZP, Piperacillin/tazobactam; SXT, Pediatric compound sulfamethoxazole tablets; SCF, Cefoperazone/sulbactam; ATM, Aztreonam; CZ, Cefazolin; CPM, Cefepime; CRO, Ceftriaxone; GM, Gentamicin; LEV, Levofloxacin; FD, Nitrofurantoin; TOB, Tobramycin; TGC, Tigecycline; PB, Polymyxin B.

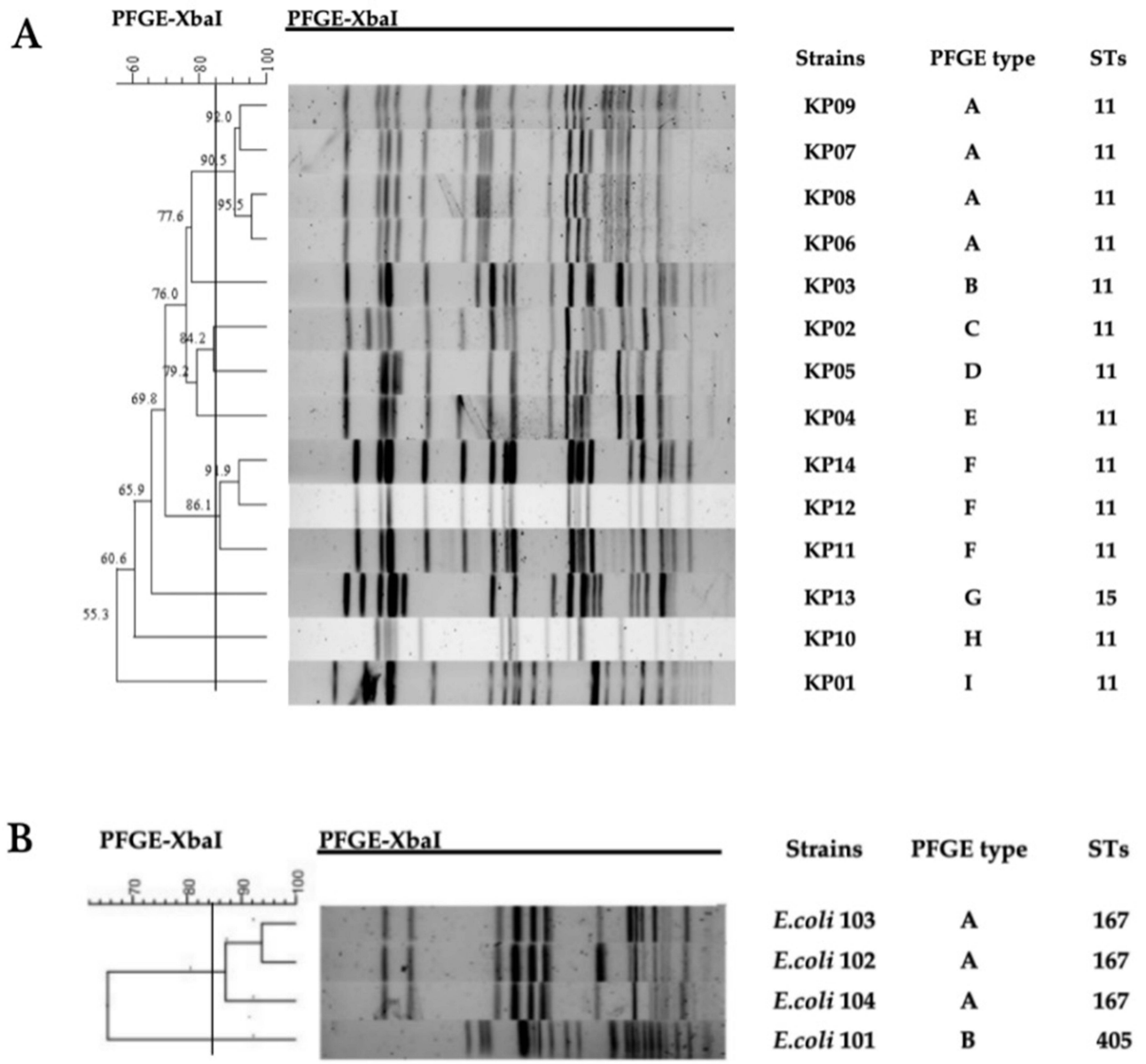

Figure 1 PFGE analysis of colistin-resistant CRE strains. (**A**) Profiles of 14 ColR-CRKP strains showing 9 clusters (AI) with ≥85% similarity. (**B**) Profiles of 4 ColR-CREC strains divided into two types, (**A**) (ST167) and (**B**) (ST405).

visualize complex phylogenetic relationships. The tree was further visualized using iTOL (Interactive Tree of Life) to highlight key features and facilitate interpretation (Figure 1).

Sequence Comparison

The *mgrB* gene was sequenced using Sanger sequencing, and mutations were identified in all 14 *K. pneumoniae* strains. PCR and sequencing analysis of ColR-CRKP strains revealed point mutations within the *mgrB* gene, with no evidence of insertion sequences. Specifically, a missense mutation A55→T, resulting in the amino acid substitution S32C, was identified in 85.7% of the isolates (n = 12; including KP01-09, KP11-12, and KP14) among the ColR-CRKP strains with a 253 bp amplicon. Additional mutations were observed in KP10 and KP13 leading to significant alterations in the amino acid sequence (Figures 2 and 3).

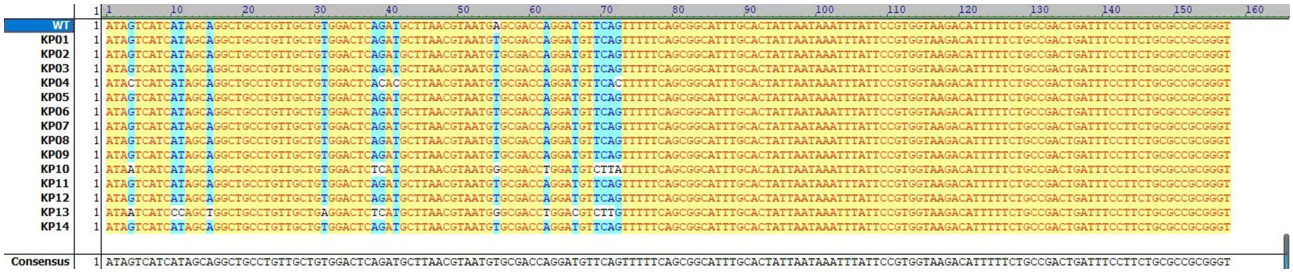

Figure 2 Sequence alignment of the *mgrB* gene among 14 ColR-CRKP strains. The sequence logos display the nucleotide composition at each position. Consensus sequence is indicated at the bottom, highlighting the conserved regions across all strains. WT (ColS-KPColS-KP-ATCC700603): colistin-sensitive *Klebsiella pneumoniae*.

Figure 3 Alignment of the protein sequences of the *mgrB* genes in 14 ColR-CRKP strains. The sequence logos display the nucleotide composition at each position. Consensus sequence is indicated at the bottom, highlighting the conserved regions across all strains. WT (ColS-KPColS-KP-ATCC700603): colistin-sensitive *Klebsiella pneumoniae*.

the *mgrB* gene mutations revealed that the A55→T mutation was present in 85.7% of isolates (n = 12), significantly higher than other mutation types. These results suggest that the A55→T mutation is a predominant factor driving colistin resistance in this region. Concurrently, the *mcr-1* gene was detected in all ColR-CREC strains.

Expression Levels of Colistin-Resistant Related Genes

Quantitative real-time PCR (qRT-PCR) analysis was performed to investigate the expression levels of genes associated with colistin resistance in colistin-resistant *Klebsiella pneumoniae* (ColR-CRKP) strains. The selected genes included *pmrA, pmrB, pmrC, pmrD, pmrE, pmrK, phoP, phoQ*, and *mgrB*. These genes are known to play roles in lipopolysaccharide modification and regulation of cell envelope stress responses, which are critical for colistin resistance. The results revealed that the expression levels of *pmrA, pmrB, pmrC, pmrD, pmrE, pmrK, phoP, and phoQ* were significantly increased, while the expression of *mgrB* was decreased in all ColR-CRKP strains compared to the colistin-sensitive wild-type strain (WT) (Table 4). These findings suggest that alterations in the expression of these genes may be associated with colistin resistance in ColR-CRKP strains. Compared to the colistin-sensitive wild-type strain (WT), the expression levels of *pmrA, pmrB, pmrC, pmrD, pmrE, pmrK, phoP, and phoQ* were significantly increased ($p < 0.05$), while the expression of *mgrB* was decreased ($p < 0.05$) in all ColR-CRKP strains.

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Table 4 Expression of Genes in Clinical Colistin-Resistant Isolates and Colistin-Sensitive Strain

Gene Name	Experimental Group Mean Expression	Control Group Mean Expression (WT)	p-value (Compared to Control)
pmrA	3.72±0.22	1.00±0.00	($p<0.05$)
pmrB	4.02±0.11	1.03±0.00	($p<0.05$)
pmrC	4.28±0.12	1.00±0.00	($p<0.05$)
pmrD	4.81±0.11	0.95±0.00	($p<0.05$)
pmrE	5.22±0.36	1.23±0.04	($p<0.05$)
pmrK	4.17±0.13	1.02±0.00	($p<0.05$)
phoP	3.41±0.12	1.01±0.00	($p<0.05$)
phoQ	3.67±0.12	0.88±0.00	($p<0.05$)
mgrB	0.07±0.01	1.74±0.05	($p<0.05$)

Notes: WT: ColS-KP-ATCC700603. The expression levels are presented as relative fold changes compared to the WT strain.

Whole Genome Sequence Analysis

E. coli 104 was chosen for whole-genome sequencing due to its representative ST and the presence of multiple resistance genes, including *mcr-1* and $bla_{\text{NDM-5}}$. *E. coli* 104, subjected to whole genome sequencing (WGS), comprised a4799433bp chromosome and four distinct-sized plasmids (160 236 bp, 111954bp, 70829bp, and95917bp). We identified 65 virulence factors in the chromosome (accession number: CP141581) and 17 antimicrobial resistance-associated genes in 4 plasmids (accession number: CP141582-CP141585). *ECO-104* plasmid p-1 (CP141582) contained several aminoglycoside-resistant genes including *aph(3")-Ib, aph(6)-Id, floR*, and a β-lactam resistant gene ($bla_{\text{TEM-1}}$), exhibiting a 98.39% identity with IncFIB (AP001918) and 96.56% identity with IncFII (pCoo) (p92944-*mph*, MG838205.1). *ECO-104* plasmid p-2 (CP141583) carried the $bla_{\text{CTX-M-199}}$ gene and exhibited 99.64% identity to IncFIB (pLF82-Phage Plasmid). *ECO-104* plasmid p-3 (CP141583) contained aminoglycoside and β-lactam resistant genes (*aadA2*, $bla_{\text{NDM-5}}$, and $bla_{\text{TEM-1}}$), showing 100% similarity to IncFII of pNDM5-IBAC (KY463220.1) and pM217-FII (NZ_AP018147.1). *ECO-104* plasmid p-4 (CP141584) contained a colistin resistance gene (*mcr-1*) and a β-lactam resistant gene ($bla_{\text{CTX-M-64}}$), displaying 98.10% similar to the IncI2 (Delta) of pBA76-*MCR*-1 (KX013540.1) and p1108-*MCR* (MG825380.1) (Figure 4). BLASTn analysis revealed high similarity between *ECO-104* plasmid p-3 and several other plasmids, namely p28078-*NDM* (MN156713.1), p_dm682b_*NDM-5* (CP095639.1), FDAARGOS_440 plasmid (CP023923.1), and pMR0617ndm (CP024039.1). Additionally, *ECO-104* plasmid p-4 displayed over 80% similarity to several plasmids from *E. coli*, such as pT28R-3 (CP049356.1), pHLJ179-167 (MN232211.1), pTBH7B1 (CP067343.1), and plasmid:1 (LR882930).

To further track bacterial sources, we analyzed *E. coli* 104 along with *mcr-1*-positive strains in BacWGSTdb. These strains underwent phylogenetic analysis based on SNP and cgMLST strategies, and a phylogenetic tree was constructed using the TVBOT online tool. The SNP-based phylogenetic tree revealed high similarity between clonal epidemic strains represented by *E. coli* 104 and ST167 strains isolated from Sichuan (CP025627, NGVI01, and WUBW01), Hangzhou (RIZW01, RIZV01, RIZU01, and RIZS01), and Shandong (RYCI01), China (Figure 5). The cgMLST-based phylogenetic tree illustrated ST167 as the predominant ST among *E. coli* isolates, followed by ST10, with distribution across humans, cows, pigs, dogs, chickens, and other species. Strains from different sources exhibited a close evolutionary relationship, suggesting the potential transmission of drug-resistant strains between humans and animals. Geographically, the ST167 epidemic strain was prevalent worldwide, especially in Latvia, India, China, and other developed countries in agriculture and animal husbandry. Although ST10 is also a global epidemic strain like ST167, it is mainly distributed in China and select developed European countries, such as Germany, the United Kingdom, and Italy (Figure 6).

Discussion

Klebsiella pneumoniae stands out among gram-negative strains in hospitals, displaying a high detection rate and posing a significant challenge in clinical settings due to its carbapenems resistance. Polymyxin, the last line of defense against

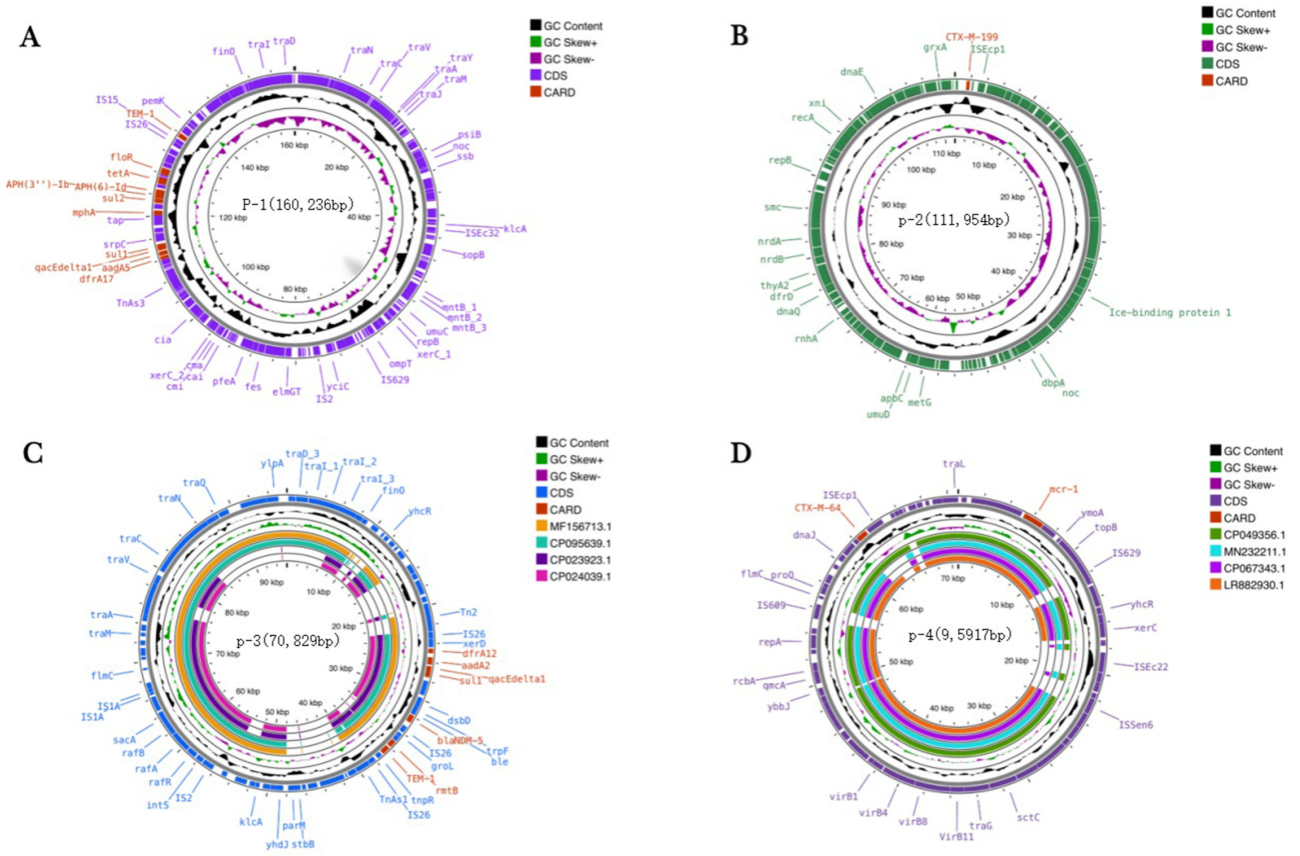

Figure 4 Circle plots and comparative analysis of plasmids carried by *E. coli* 104. (**A**) Circle plots of *ECO-104* plasmid p-1 (160,236bp). (**B**) Circle plots of *ECO-104* plasmid p-2(111,954 bp). (**C**) Comparative analysis of *ECO-104* plasmid p-3(70,829 bp) with other 4 plasmids (p28078-*NDM*, p_dm682b_*NDM-5*, FDAARGOS_440 plasmid, and pMR0617ndm). (**D**) Comparative analysis of *ECO-104* plasmid p-4(9,5917 bp) with other 4 plasmids (pT28R-3, pHLJ179-167, pTBH7P1, and plasmid:1). The outermost circle represents plasmid sequences in this study.

multi-drug-resistant *K. pneumoniae*, encounters resistance, and recent studies report the emergence of polymyxin resistance in *K. pneumoniae* in several countries, with the ST258 type being the most prevalent clonal type.[15] Two-component regulatory systems play an important role in the modification of LPS, such as the inactivation of *mgrB* regulators caused by insertion sequence leading to the development of polymyxin resistance,[16] which was speculated to be the main mechanism of *K. pneumoniae*.[17]

In our study, all 14 K. *pneumoniae* strains exhibited mutations in the *mgrB* gene. Nucleotide sequence comparisons revealed that *mgrB* gene mutations occurred mostly 85.7% (12/14) in A-base mutations to T at the 55 locus, resulting in an amino acid sequence change (S32C). The *mgrB* mutation (A55→T, resulting in S32C) identified in our study is consistent with global reports on polymyxin-resistant *K. pneumoniae* strains.[18–20] This mutation significantly impacts colistin resistance by disrupting the PhoP-PhoQ regulatory system, leading to LPS modification and reduced polymyxin binding.[7] The mutated strains showed significantly decreased expression of the *mgrB* gene compared to the wild-type strain, and the MIC of the corresponding strains to polymyxin increased to over 8 mg/L, indicating high-level polymyxin resistance.

Although mutations in bases are mostly random, certain triggers have been reported to increase the chance of mutation, such as the use of antimicrobial drugs. From the clinical data of patients infected with ColR-CRKP strains, it was observed that most of them had a history of antimicrobial drug use, such as third-generation cephalosporin and enzyme inhibitor (14/14, 100%), and carbapenems (8/14, 57.1%). This frequent exposure to antibiotics may have contributed to the selection of *mgrB* mutations, although a direct causal link remains elusive.[21]

Notably, In our study, all 14 *K. pneumoniae* strains exhibited mutations in the *mgrB* gene without inactivation by insertion sequences, differing from previous studies, especially in other countries.[18] This difference may be attributed to

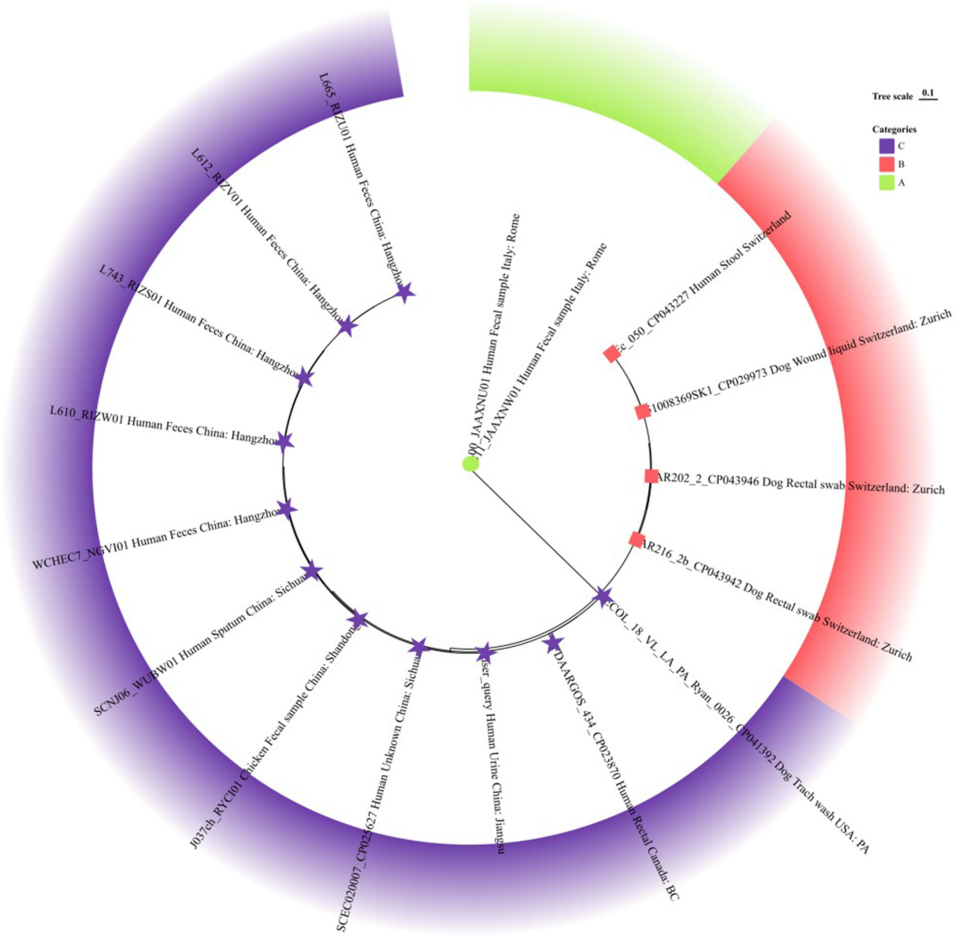

Figure 5 Phylogenetic tree based on the SNP strategy of *E. coli* 104. The same colors represent a closer evolutionary relationship. *E. coli* 104 in this study was assigned to category C.

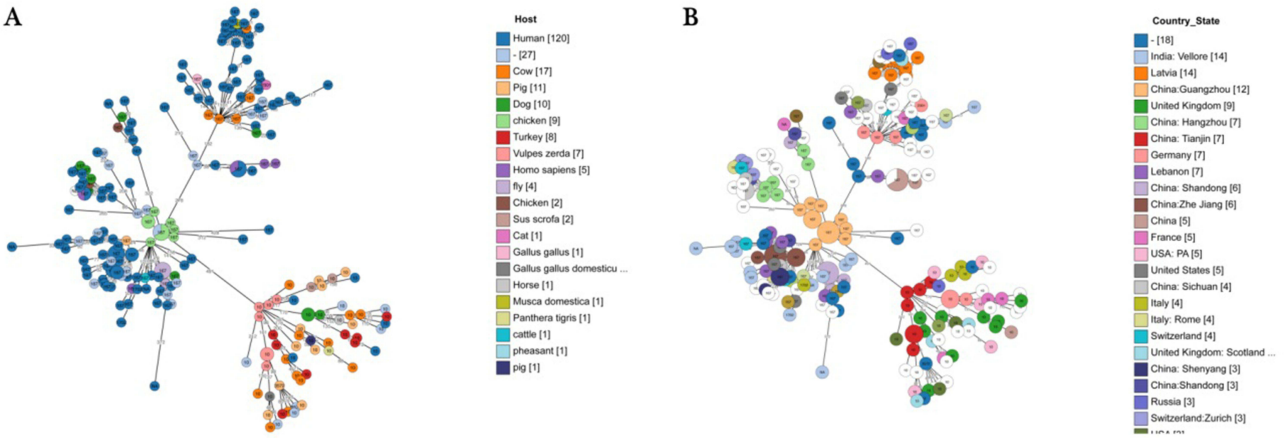

Figure 6 Phylogenetic tree based on the cgMLST strategy of *E. coli* 104 in this study with *mcr-1*-positive strains in the BacWGSTdb. (**A**) Phylogenetic tree based on host. Different colors represent different hosts. The numbers in the circle represent the ST types. (**B**) Phylogenetic tree based on region. Different colors represent different regions. The numbers in the circle represent the ST types.

the specific clinical practices and antibiotic usage patterns in our region, which warrant further investigation. Therefore, mutations in the *mgrB* gene were likely a result of these antimicrobial drugs; however, further validation of the mechanism is required.

Our study observed varying levels of colistin resistance among strains with different *mgrB* mutations. The A55T mutation, resulting in the S32C amino acid substitution, was associated with high-level resistance (MIC ≥ 16 mg/L) in 12 out of 14 isolates (85.7%), while other mutations, such as A55G, exhibited lower resistance levels (MIC = 8 mg/L) in the remaining 2 isolates (14.3%). revealed significant differences in colistin resistance levels among strains with different *mgrB* mutations. These findings highlight the need for further investigation into the specific impacts of different *mgrB* mutations on resistance mechanisms and are consistent with previous reports[17], emphasizing the importance of *mgrB* mutations in colistin resistance.

While our study suggests that *mgrB* gene mutations may be associated with the use of antimicrobial drugs, as reported in other studies,[7,18] a definitive cause-and-effect relationship has not been established. Although most patients with *mgrB* mutations had a history of antibiotic use, a direct causal link between antibiotic exposure and *mgrB* mutations remains elusive. However, studies have suggested that certain antibiotics, such as carbapenems, may indirectly promote *mgrB* mutations by inducing genetic changes or selecting for resistant strains.[16,17] Further research is necessary to elucidate the specific mechanisms by which antibiotics contribute to the development of these mutations. This could involve longitudinal studies to track the emergence of *mgrB* mutations in patients receiving different antibiotic regimens, or experimental studies to investigate the genetic and environmental factors that drive these mutations.

Another significant mechanism of colistin resistance is the presence of the *mcr* gene. The discovery of *mcr-1* as a plasmid-mediated determinant of colistin resistance in China has garnered global attention.[22–24] While there are ten *mcr* gene family variants (*mcr-1* to *mcr-10*), *mcr-1* is typically considered the primary mechanism of polymyxin resistance, especially in *E. coli*.[25–27] In our study, we collected four strains of polymyxin-resistant *E. coli*, including two strains from urine and two strains from blood. Moreover, these patients had no history of polymyxin use but had used other antibiotics. The presence of *mcr-1* led to a low level of resistance to colistin (MIC = 4 mg/L), which is consistent with the literature worldwide reports on *mcr-1*-mediated polymyxin resistance in *E. coli*.[28,29] However, further studies are needed to explore potential co-resistance to other antibiotics and other factors influencing resistance.

To place our findings within the broader global context of *mcr-1*-mediated resistance, a comparison with other studies is essential. Such comparisons will help us understand how our results fit into the global landscape of polymyxin resistance. The low-level resistance mediated by *mcr*-1 (MIC = 4 mg/L) has been widely reported globally.[22] However, the low-level resistance observed in this study may be associated with the coexistence of other resistance genes, which warrants further investigation. Our study's *mcr-1* positive isolates exhibited high genetic similarity with strains from other regions, such as India and China. For example, *E. coli* 104 clustered closely with ST167 strains from these regions in the phylogenetic tree, indicating a shared evolutionary origin. This finding aligns with global reports on the spread of *mcr-1* mediated resistance.[24]

Future studies should include multicenter investigations to validate the prevalence of *mgrB* mutations and *mcr-1* genes in diverse clinical settings. Comparative genomic analyses of additional isolates, such as *E. coli* 101 and *E. coli* 104, are recommended to elucidate genetic differences and their functional implications. These efforts will enhance our understanding of the mechanisms underlying polymyxin resistance and inform targeted interventions. Given the limitations identified in our study, additional validation through whole-genome sequencing (WGS) and comprehensive genomic comparisons is essential. Such studies will not only validate the findings but also provide deeper insights into the genetic and functional differences between strains, ultimately guiding more effective strategies to combat antimicrobial resistance.

Our whole-genome sequencing (WGS) analysis of *E. coli* 104, a representative ST167 strain harboring *mcr-1*, revealed critical insights into the genetic architecture of colistin resistance. The strain carried four plasmids, each encoding distinct resistance determinants: p-1 (IncFIB/IncFII): Contained *aph(3")-Ib, aph(6)-Id, floR*, and $bla_{\text{TEM-1}}$, conferring resistance to aminoglycosides and β-lactams. p-2 (IncFIB): Encoded $bla_{\text{CTX-M-199}}$, an extended-spectrum β-lactamase (ESBL). p-3 (IncFII): Harbored $bla_{\text{NDM-5}}$ and $bla_{\text{TEM-1}}$, mediating carbapenem and penicillin resistance. p-4 (IncI2): Carried *mcr-1* and $bla_{\text{CTX-M-64}}$, pivotal for colistin and cephalosporin resistance.

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Notably, *mcr-1* and $bla_{\text{NDM-5}}$ were located on separate plasmids (IncI2 and IncFII, respectively), suggesting independent acquisition events. This genetic arrangement facilitates horizontal transfer of resistance genes across bacterial populations. Comparative analysis revealed >98% similarity between p-3 and globally circulating $bla_{\text{NDM-5}}$ -plasmids (eg, p28078-*NDM* [MN156713.1], pMR0617ndm [CP024039.1]), indicating transregional dissemination of carbapenem resistance. Similarly, p-4 (*mcr-1*-bearing IncI2 plasmid) shared high identity (>98%) with plasmids from clinical and environmental *E. coli* strains in China (eg, pBA76-*MCR-1* [KX013540.1]), underscoring the role of mobile genetic elements in perpetuating colistin resistance.

Phylogenetic analysis further demonstrated that *E. coli* 104 clustered closely with ST167 strains from Sichuan, Hangzhou, and Shandong (China), as well as international isolates from Latvia and India. This clonal relatedness, supported by both SNP and cgMLST trees, highlights the potential for cross-regional and cross-species spread of *mcr-1*-harboring strains. The detection of ST167 in humans, livestock (cows, pigs, chickens), and companion animals (dogs) aligns with the One Health approach, emphasizing how agricultural antibiotic use and zoonotic transmission contribute to resistance dissemination.

These findings underscore two critical public health concerns: Co-resistance threats: The coexistence of *mcr-1*, $bla_{\text{NDM-5}}$, and ESBL genes on plasmids creates pan-drug-resistant phenotypes, leaving few therapeutic options. Epidemiological linkages: Clonal expansion of ST167 *E. coli* across China and beyond necessitates enhanced surveillance to track resistance gene flow at the human-animal-environment interface.

Our phylogenetic analysis revealed close evolutionary relationships between *mcr-1*-positive strains from humans and animals, underscoring the importance of the One Health approach. The interplay between human health, animal health, and environmental factors likely facilitates the spread of resistant strains. For instance, the extensive use of antibiotics in agriculture and livestock farming may contribute to the emergence and dissemination of resistant bacteria. Future research should explore these interactions more thoroughly to develop comprehensive strategies for controlling antimicrobial resistance. This includes stringent antibiotic management measures in both clinical treatment and agricultural settings, as well as a deeper understanding of how these components interact to facilitate the spread of resistant strains.

Limitations

This study has several limitations that should be acknowledged. Firstly, the small sample size (n=18) may limit the generalizability of the findings. A larger sample would provide more robust statistical power and enhance the reliability of the results. Secondly, the lack of control strains from the environment means that we cannot fully account for potential external factors that may have influenced the outcomes. Lastly, the retrospective nature of the patient data may introduce biases related to data collection and recall, which could affect the accuracy and completeness of the information used in the analysis.

Conclusions

This study presents two distinct mechanisms contributing to colistin resistance in Enterobacterales. Mutations in the *mgrB* gene and the plasmid-carried *mcr-1* gene account for colistin resistance in *K. pneumoniae* and *E. coli,* respectively. The acquisition of *mcr-1* by *K. pneumoniae* with an existing *mgrB* gene mutation poses a substantial clinical challenge, as it results in a more resistant Enterobacterales. Whole-genome sequencing (WGS) revealed the coexistence of multiple resistance genes, such as *mcr-1* and $bla_{\text{NDM-5}}$, in some strains. This coexistence suggests that these strains may have a broader resistance profile, complicating treatment options. Additionally, the presence of these genes on plasmids indicates a potential for horizontal transfer, which could further spread resistance. Future studies should focus on understanding the genetic mechanisms underlying the coexistence of these resistance genes and their potential impact on clinical outcomes. Additionally, the phylogenetic analysis highlighted a close evolutionary relationship between *mcr-1*-positive strains isolated from both humans and animals, underscoring the potential for cross-species transmission. Given these findings, there is an urgent need to strengthen the epidemiological surveillance of drug-resistant bacteria and reinforce the management system of the rational use of antibiotics.

Data Sharing Statement

The sequence data have been submitted to NCBI database (accession number: CP141581-CP141585).

Ethics Approval and Consent to Participate

All strains were isolated from culture samples collected for routine clinical examinations of hospitalized patients admitted to the Affiliated Hospital of Xuzhou Medical University between May 2016 and June 2022. Any personally identifiable information was removed from this study. This study protocol, including the waiver of informed consent due to the use of anonymized data from routine clinical practice, was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2021-KL101-01). The research involved no more than minimal risk to subjects and no personal information was obtained. The research conformed to the principles of the Helsinki Declaration.

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Disclosure

The authors declare no conflicts of interest in this work.

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