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Emerging Problems in Infectious Diseases

Detection of mcr-1 harbouring multidrug-resistant Escherichia coli from hospitalized patients and aquatic environments

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Abstract

Introduction: The global dissemination of colistin resistance poses a critical challenge to antimicrobial therapy amid increasing multidrug resistance. The objective of this study was to characterize the epidemiology and transmission of colistin resistance mediated by the *mcr-1* gene in multidrug-resistant (MDR) *Enterobacteriaceae* isolated from hospitalized patients with bloodstream infections and adjacent aquatic environments.

Methodology: A three-year study was conducted to collect MDR *Enterobacteriaceae* isolates from two distinct sources: blood samples and environmental water samples from five distinct aquatic environments adjacent to the hospital. A systematic analysis was conducted on patient demographic data, bacterial identification, antimicrobial susceptibility profiles, *mcr-1* gene screening, and conjugation assays.

Results: A total of 159 MDR *Enterobacteriaceae* were isolated from blood samples, demonstrating an overall colistin resistance rate of 4.4%. Three *mcr-1*-harbouring *Escherichia coli* (*E. coli*) strains were identified: one from an inpatient without colistin exposure, and two from aquatic environmental sources, classified into sequence types (STs) ST10, ST131, and ST155. Conjugation experiments revealed higher transfer frequencies for environmental *mcr-1*-positive *E. coli* strains within and across genera. A comparative analysis under varying nutrient conditions identified delayed transfer kinetics in aquatic isolates. Moreover, most transconjugants exhibited levels of colistin resistance that were comparable to or surpassing donor strains, with minimum inhibitory concentrations (MICs) ranging from 4 to 16 mg/L.

Conclusions: This study documents the epidemiological patterns of *mcr-1*-mediated colistin resistance in clinical and aquatic MDR *Enterobacteriaceae*. The present findings indicate the dissemination of resistance traits within aquatic environments, where persistence and amplification were observed.

Key words: Colistin resistance; *mcr-1* gene; *Escherichia coli*; bloodstream infections; aquatic environments; conjugation.

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Introduction

Enterobacteriaceae represent predominant pathogens in nosocomial bloodstream infections (nBSIs) [1]. *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are highly prevalent causative agents that exhibit elevated rates of antimicrobial resistance across multiple regions. A multitude of studies have reported an escalating prevalence of multidrug resistance (MDR) among *Enterobacteriaceae* [2-4]. The prevalence of carbapenem resistance is a particularly salient concern: Jiang *et al.* documented imipenem resistance in ≤ 50% of *K. pneumoniae* isolates [5], while a 12-year national study from the USA revealed a twofold increase in carbapenem-resistant *Enterobacteriaceae* [6]. Infections caused by MDR *Enterobacteriaceae* contribute to poor clinical outcomes due to limited therapeutic options. This scarcity has prompted a re-evaluation of using colistin to treat severe MDR *Enterobacteriaceae* infections.

Colistin, a cationic antibiotic, disrupts bacterial membrane permeability by targeting lipid A in lipopolysaccharides (LPS) through electrostatic interactions, ultimately leading to bacterial death [7]. However, its clinical use was largely discontinued in the 1970s due to concerns regarding its neurotoxicity and nephrotoxicity. Subsequently, it was widely utilized as a growth promoter in animal husbandry. In recent years, the plasmid-mediated colistin resistance gene, *mcr-1*, has been identified in *Enterobacteriaceae* from diverse sources, including animals, humans, and the environment. Effluents discharged from households, hospitals, industries, and agricultural operations contain antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs). Horizontal gene transfer via conjugation is a significant pathway for disseminating ARGs [8-13]. Recent surveys have revealed the presence of the *mcr-1* gene in various aquatic environments [14]. Furthermore, elevated levels of colistin resistance have been observed following its reintroduction into clinical use in 2017 [15]. The increasing prevalence of the *mcr-1* gene and

colistin resistance may contribute to the emergence of pan-resistant bacteria, which poses a substantial threat to public health. Consequently, monitoring the prevalence of colistin resistance and the *mcr-1* gene is essential to enhance understanding of their epidemiology and transmission dynamics. In view of the aforementioned concerns, a three-year study was conducted to investigate the prevalence of colistin resistance and the *mcr-1* gene among MDR *Enterobacteriaceae* isolated from blood cultures of inpatients from 2020 to 2023. The extended evidence suggests the presence of the *mcr-1* gene in aquatic environments, thus indicating that environmental water may serve as a potential transmission route. By extending analysis to aquatic environments near the hospital, this study aims to trace transmission routes while examining dynamics of colistin resistance mediated by the *mcr-1* gene across clinical (inpatient) and environmental reservoirs.

Methodology

Study design

A retrospective study was conducted at Nanjing Pukou People's Hospital (Liangjiang Hospital, Southeast University) in Nanjing, China, from January 2020 to January 2023. Patients admitted to the hospital were included for the investigation of the mcr-1 gene and colistin resistance during the study period. nBSI was defined as the first positive blood culture obtained ≥ 48 hours on hospital admission in accordance with the CDC definition of microbiologically documented BSI. The clinical data and medical records were reviewed with the approval of the Ethics Committee of Nanjing Pukou People's Hospital (Liangjiang Hospital, Southeast University). All isolates from patients were obtained from routine laboratory tests, and no additional samples were taken from the patients.

Specimen collection and identification

Collection of clinical isolates: From January 2020 to January 2023, all MDR *Enterobacteriaceae* isolates from nBSIs in hospitalized adults (aged ≥ 18 years) were included in the study. MDR *Enterobacteriaceae* were defined as *Enterobacteriaceae* isolates resistant to at least one antimicrobial agent in three or more antimicrobial groups. The identification of all isolates was conducted using the VITEK 2 system (BioMerieux, France).

Environmental sampling: A total of five sites along the Chengnan River adjacent to the hospital were selected for the collection of complementary water samples (see Supplementary Figure 1). At each site, triplicate 10 mL surface water samples (extracted from a depth of 10 cm) were obtained in sterile tubes. Following transport to the laboratory, samples were plated on colistin-containing MacConkey agar (2 mg/L; Oxoid, UK) and incubated overnight at 37 °C. The selection of presumptive colistin-resistant *Enterobacteriaceae* was based on colony morphology and confirmed through VITEK 2 identification and antimicrobial susceptibility testing.

Antimicrobial susceptibility and heteroresistance to colistin

All susceptibility tests, except for colistin, were performed using the VITEK-2 system. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards (M100 31st, 2021). Extended-spectrum β-lactamases (ESBLs) were confirmed by the double disk synergy test. The quality control strains included *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. pneumoniae* 700603, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, and *Acinetobacter baumannii* (*A. baumannii*) ATCC BAA-747.

Colistin resistance was determined among bloodstream isolates enrolled in this study by the broth microdilution method, in accordance with the guidelines established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The MIC values were interpreted as susceptible at ≤ 2 mg/L and resistant at > 2 mg/L.

Colistin heteroresistance in colistin-susceptible bloodstream isolates was assessed using population analysis profiles (PAPs) as previously described [16]. Isolates were cultured to the logarithmic growth phase and adjusted to 0.5 McFarland. Aliquots (50 μL) of each suspension were plated onto MH agar containing two-fold colistin dilution (0–16 mg/L; 0, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/L). Following 24-hour incubation at 37 °C, colonies were enumerated within the quantifiable range (20-200 CFU/mL). Isolates exhibiting colistin-resistant subpopulations growing at ≥ 2 mg/L colistin were classified as heteroresistant. These strains underwent ten antibiotic-free passages with subsequent retesting to determine resistance stability.

Isolates exhibiting resistance or heteroresistance to colistin were collected for further detection of the *mcr-1* gene.

The mcr-1 screening and MLST analysis

Colistin non-susceptible *Enterobacteriaceae* from aquatic environments and bloodstreams specimens underwent *mcr-1* screening. Genomic DNA was

extracted using boiling lysis method. Multiplex quantitative real-time PCR (qPCR) amplified the target *mcr-1* gene and *16SrRNA* reference gene. For qPCR-positive samples, conventional PCR with *mcr-1*-specific primers (F: 5'-CAAACCTATCCCATCGCGGA-3'; R: 5'-AGCTGAACATACACGGCACA-3') generated amplicons for sequence verification. PCR products corresponding to the *mcr-1* gene from positive *E. coli* strains (clinical isolate ENT-P1, aquatic isolates ENT-W1 and ENT-W3) were purified and sequenced by Sanger's methodology. The resulting sequences were deposited in GenBank under accession numbers PV551116 (ENT-P1), PV551117 (ENT-W1), and PV551118 (ENT-W3).

For molecular typing, multilocus sequence typing (MLST) was performed by amplifying seven *E. coli* housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA,* and *recA*) following established protocols. Amplicons underwent Sanger sequencing (accessions: ENT-W1: PV604350-PV604356; ENT-W3: PV604357-PV604363; ENT-P1: PV604364-PV604370). Sequence types were determined using the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Complete details concerning the PCR are provided in Supplementary Table 1.

Bacterial conjugations under different culture conditions within and across genera

Two conjugation systems, designated A and B, were established to evaluate the *mcr-1* gene transfer under varying culture conditions and between bacterial genera. In system A, the transfer of *mcr-1* was assessed using three donor strains: *E. coli* ENT-P1 (clinical), ENT-W1 (aquatic), and ENT-W3 (aquatic). Sodium azide (NaN3)-resistant *E. coli* J53 (Bioscibio, China) functioned as the intrageneric recipient, while colistin-susceptible *K. pneumoniae* 700603 served as the intergeneric recipient.Overnight cultures were centrifuged, washed, and resuspended to a concentration of 1×10^8 CFU/mL. After mixed-broth conjugation, transconjugants were selected on two types of media. For intrageneric transfer, MH agar containing 2 mg/L colistin and 200 mg/L NaN3 was used. For intergeneric transfer, the same medium was supplemented with 30 mg/L of cefotaxime. The conjugation frequencies were calculated as the number of transconjugants per recipient. Colistin resistance was confirmed by broth microdilution.

To simulate bacterial conjugations in aquatic environments and explore the influence of growth conditions, two sets of conditions were established in parallel for conjugation system B. The first set used routine nutrient conditions (LB broth). The second set of conditions is representative of the aquatic environment. Environmental waters were collected from the designated sampling sites mentioned above. These waters were homogenized, heat-sterilized, and filtered through 0.45 μm membranes to remove bacteria that might confound the conjugation experiments. After preparation, procedures for the conjugation system B were performed. Mating mixtures were incubated statically at 28 °C in both LB broth and environmental waters, mimicking natural aquatic environments. After incubation, samples were collected at 12-hour intervals and inoculated on MH plates supplemented with selected agents as described for system A (2 mg/L colistin plus 200 mg/L NaN3 for intrageneric selection, 2 mg/L colistin plus 30 mg/L cefotaxime for intergeneric selection) to isolate the transconjugants.

Statistical analysis

Data were analyzed by SPSS software version 15.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism software (San Diego, USA). Continuous data were expressed as mean ± standard deviation and analyzed by Student's t-test. Categorical data were expressed as frequencies and percentages and analyzed by the Chi-square test. One-way analysis of variance (ANOVA) and the Wilcoxon test were employed to compare conjugative transfer frequencies. A p of less than 0.05 was considered statistically significant.

Table 1. Antibiotic resistant profiles among MDR *Enterobacteriaceae*.

Strains	Numbers of antibiotic resistance (N)													
	COL	AM	TZP	ATM	CAZ	FEP	CRO	GM	AK	TOB	CIP	LEV	MEM	IPM
E. coli	3	71	16	41	44	32	55	34	16	32	58	52	11	10
K. pneumoniae	2	28	20	26	25	20	21	15	10	14	27	25	12	15
P. aeruginosa	0	12	6	7	7	3	5	5	2	4	10	10	11	11
A. baumanii	0	18	17	19	19	19	17	14	13	17	16	15	17	17
E. cloacae	1	6	1	4	3	2	4	2	1	5	4	5	1	2
Salmonella	1	1	1	1	1	1	2	1	1	1	1	1	1	1
Other Klebsiella	0	2	3	3	2	4	4	2	1	3	4	4	1	2
Total (%)	7 (4.4)	138 (86.8)	64 (40.3)	101 (63.5)	101 (63.5)	81 (50.9)	108 (67.9)	73 (45.9)	44 (27.7)	76 (47.8)	120 (75.5)	112 (70.4)	54 (34.0)	58 (36.5)

COL: colistin; AM: ampicillin; TZP: piperacillin/tazobactam; ATM: aztreonam; CAZ: ceftazidime; FEP: cefepime; CRO: ceftriaxone; GM: gentamicin; AK: amikacin; TOB: tobramycin; CIP; ciprofloxacin; LVX: levofloxacin; MEM: meropenem; IMP: imipenem. All MICs were interpreted in accordance with the CLSI guidelines: with the exception of colistin: which followed the EUCAST guidelines.

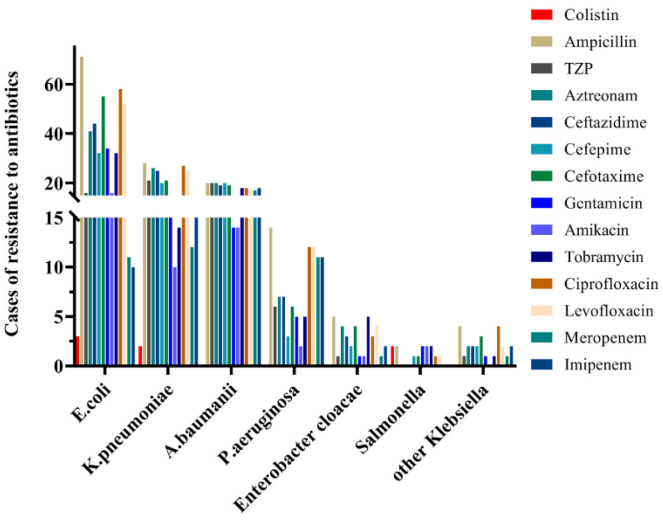
Results

Clinical information on bloodstream isolates of MDR Enterobacteriaceae

A total of 159 nonrepetitive *Enterobacteriaceae* were isolated from 159 inpatients with nBSIs, including 95 (59.7%) male and 64 (40.3%) female patients. Most patients exhibited varying degrees of underlying diseases or conditions. Among these patients, 67 (42.1%) were receiving intensive care, with 41 (25.8%) in the intensive care unit (ICU) and 26 (16.3%) in pulmonary and critical care medicine (PCCM). Others were from the hematology department (20.8%) and the oncology ward (8.2%).

Regarding the *Enterobacteriaceae* isolates, *E. coli* (n = 77, 48.4%) was the predominant species, followed by *K. pneumoniae* (n = 31, 19.5%) and *A. baumannii* (n = 22, 13.8%). The remaining isolates were *P. aeruginosa* (n = 16, 10.1%), *Enterobacter cloacae* (*E. cloacae*) (n = 7, 4.4 %), and *Salmonella* (n = 2, 1.3%).

Figure 1. Antimicrobial resistance profile of bloodstream isolates to 14 different antibiotics from January 2020 to January 2023.



Number of resistances to 14 antibiotics among the isolates obtained from the bloodstream. Other Klebsiella refers to Klebsiella aerogenes, Klebsiella oxytoca, and other opportunistic Klebsiella species.

Table 2. Clinical data of patients with MDR Enterobacteriaceae infection.

Characteristic	Colistin non-susceptible (N = 12)	Colistin susceptible (N = 147)	*p*	F/Z
Gender			**0.29**	**1.133**
Male (n = 95)	8 (66.7)	87 (59.2)		
Female (n = 64)	4 (33.3)	60 (40.8)		
Age			**0.729**	**0.121**
Mean	65.67 ± 15.93	67.98 ± 15.69		
Median (min, max)	62.5 (39,86）	70 (25,100）		
Ward, n (%)			**0.044**	**12.926**
ICU	1 (8.3)	40 (27.2)		
Hematology	6 (50)	27 (18.4)		
Oncology	1 (8.3)	12 (8.2)		
Neurosurgery	0 (0)	6 (4.1)		
PCCM	4 (33.3)	22 (15.0)		
Cardiovascularology	0 (0)	11 (7.5)		
Others	0 (0)	29 (19.7)		
Mechanical ventilation	3 (25.0)	50 (34.0)	0.775	0.510
LOS (days)	40.25 ± 24.92	18.13 ± 14.44	0.019	5.664
28-day mortality	0 (0)	3 (2.0)	0.412	0.710
Bacteria			0.012	17.924
E. coli	3 (25.0)	74 (50.3)		
K. pneumoniae	5 (41.7)	26 (17.7)		
A. baumannii	1 (8.3)	21 (14.3)		
P. aeruginosa	1 (8.3)	15 (10.2)		
E. cloacae	1 (8.3)	6 (4.1)		
Salmonella	1 (8.3)	1 (0.7)		
Other Klebsiella	0 (0)	4 (2.7)		
MDR/XDR strains			**0.586**	**0.296**
MDR	11 (91.7)	140 (95.2)		
XDR	1 (8.3)	7 (4.8)		
Resistance type			**0.675**	**2.329**
ESBL	3 (25.0)	54 (36.7)		
CRE	5 (41.7)	47 (32.0)		

ICU: intensive care unit; PCCM: pulmonary and critical care medicine; LOS: length of hospital stay; MDR: multidrug resistant; XDR: extensively drug resistant; ESBL: extended-spectrum β-lactamases-producing bacteria; CRE: carbapenem-resistant bacteria. Age and LOS were analyzed by Student's t-test. The numbers in parentheses represent percentages were analyzed by Chi-square test.

Table 3. Antimicrobial susceptibility profiles of colistin-resistant isolates from different origins.

Sources	MICs (mg/L)															
	ID	Strains	COL	AM	TZP	ATM	CAZ	FEP	CRO	GM	AK	TOB	CIP	LEV	MEM	IPM
Bloodstream	ENT-P1	*E. coli*	4	≥ 32 (R)	≤ 4 (S)	2 (S)	32 (R)	≥ 32 (R)	≤ 1 (S)	≥ 16 (R)	≥ 64 (R)	≤ 4 (S)	≥ 1 (R)	≥ 2 (R)	≥ 4 (R)	≤ 0.25 (S)
Aquatic environment	ENT-W1	*E. coli*	4	≥ 32 (R)	≤ 4 (S)	2 (S)	≥ 64 (R)	≥ 32 (R)	≤ 1 (S)	≥ 16 (R)	≥ 64(R)	≤ 4 (S)	≤ 0.25 (S)	≤ 0.5 (S)	≤ 0.25 (S)	≤ 0.25 (S)
	ENT-W2	*E. coli*	4	≤ 8 (S)	≤ 4 (S)	≤ 1 (S)	4 (S)	8 (S)	≤ 1 (S)	≤ 4 (S)	≤ 16 (S)	≤ 4 (S)	≤ 0.25 (S)	≤ 0.12 (S)	≤ 0.25 (S)	≤ 0.25 (S)
	ENT-W3	*E. coli*	8	≥ 32 (R)	≤ 4 (S)	≥ 64 (R)	≥ 64 (R)	≥ 32 (R)	≥ 4 (R)	≥ 16 (R)	≥ 64 (R)	≥ 16 (R)	≥ 1 (R)	≥ 2 (R)	≤ 0.25 (S)	≥ 4 (R)
	ENT-W4	*E. coli*	4	≥ 32 (R)	≤ 4 (S)	2 (S)	4 (S)	≤ 1 (S)	≤ 1 (S)	≥ 16 (R)	≥ 64 (R)	≤ 4 (S)	≤ 0.25 (S)	0.25 (S)	≤ 0.25 (S)	0.5 (S)
	ENT-W5	*E. coli*	4	≥ 32 (R)	≤ 4 (S)	16 (R)	16 (R)	≥ 32 (R)	≥ 4 (R)	≥ 16 (R)	≥ 64 (R)	≤ 4 (S)	≤ 0.25 (S)	≤ 0.12 (S)	≤ 0.25 (S)	≤ 0.25 (S)
	ENT-W6	*E. coli*	4	≥ 32 (R)	≤ 4 (S)	2 (S)	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)	≤ 4 (S)	≥ 64 (R)	≥ 16 (R)	≤ 0.25 (S)	≤ 0.5 (S)	≤ 0.25 (S)	≤ 0.25 (S)
	ENT-W7	*E. coli*	8	≤ 8 (S)	≤ 4 (S)	2 (S)	32 (R)	≤ 1 (S)	≤ 1 (S)	≤ 4 (S)	≤ 16 (S)	≤ 4 (S)	≤ 0.25 (S)	≤ 0.5 (S)	≥ 4 (R)	≤ 0.25 (S)
	ENT-W8	*C. freundii*	4	≥ 32 (R)	64 (R)	≤ 1 (S)	4 (S)	≤ 1 (S)	≤ 1 (S)	≤ 4 (S)	≤ 16 (S)	≤ 4 (S)	≤ 0.25 (S)	≤ 0.12 (S)	≤ 0.25 (S)	≤ 0.25 (S)

R: resistant; S: susceptible.

Among all 159 isolates, 57 (35.8%) were confirmed as ESBL producers, and 52 (32.7%) as carbapenem-resistant *Enterobacteriaceae* (CRE). High incidences of resistance were observed to ampicillin (86.8%), ciprofloxacin (75.5%), levofloxacin (70.4%), ceftriaxone (67.9%), ceftazidime (63.5%), aztreonam (63.5%), and cefepime (50.9%) (Table 1). Figure 1 illustrates the distribution of these isolates across antibiotics. Colistin resistance was found in 7 isolates (4.4%). Of the colistin-susceptible isolates (152, 95.6%), five (5/152, 3.3%) displayed resistant subpopulations (colistin MIC range: 4–16 mg/L) by PAPs, including four *K. pneumoniae*, and one *A. baumannii*. After ten generations of subculture, only one *A. baumannii* isolate maintained a consistent colistin MIC of 4 mg/L (Supplementary Table 2). There were no statistically significant differences in age or gender between the colistin non-susceptible group and the colistin susceptible group ($p > 0.05$). However, patients in the colistin non-susceptible group had a longer hospital stay (40.25 ± 24.92) ($p = 0.019$) (Table 2).

A multiplex qPCR was conducted on all colistin non-susceptible isolates (colistin-resistant and heteroresistant isolates). Of the isolates tested, one *E. coli* strain (1/12) isolated from an 81-year-old patient in the ICU was identified as colistin-resistant and *mcr-1* positive (denominated ENT-P1). The patient had no documented history of exposure to colistin or polymyxin. Antibiotic susceptibility tests demonstrated that this *mcr-1*-carrying strain exhibited extended resistance to most commonly used antibiotics (Table 3). Given the established role of aquatic environments as a significant source of pathogens, antibiotics, and antimicrobial resistance genes (ARGs), it is imperative to elucidate the transmission patterns of *mcr-1* and enhance the epidemiological information within our district. To this end, waterborne isolates in the vicinity of the hospital were collected for *mcr-1* gene screening and further analysis.

Antimicrobial resistance and mcr-1 detection among waterborne Enterobacteriaceae

Fifteen water samples were collected from five distinct sites along the Chengnan River in the vicinity of the hospital, and eight samples displayed colistin resistance (MIC range: 4-16 mg/L). These isolates were identified as seven *E. coli* and one *C. freundii.* A significant proportion (6/8) of the multidrug-resistant isolates was identified. The most prevalent resistance was to ampicillin (75%, 6/8), followed by the aminoglycosides with resistance rates ranging from 50% to 62.5%. Third-generation cephalosporins exhibited a resistance rate range of 25% to 50%, while carbapenems demonstrated a lower resistance rate of 12.5%, as illustrated in Table 3. Among the colistin-resistant isolates, two *E. coli* isolates (ENT-W1 from site 1 and ENT-W3 from site 3) presented *mcr-1* positive. These findings confirmed the presence of the *mcr-1* gene in environmental water sources in the vicinity of the hospital. A previous study from Nanjing Tech University reported high concentrations of *mcr-1* on the North Bank of the Yangtze River, including the Chengnan River, which is consistent with the findings of the present study [17].

Sequence types of mcr-1-gene-carrying strains from different origins

MLST was employed on three *mcr-1*-positive strains obtained from the bloodstream and aquatic environments. The results of the MLST analysis revealed that these isolates from disparate origins were genetically unrelated, with sequence types identified as ST10 (ENT-P1), ST131 (ENT-W1), and ST155 (ENT-

Table 4. Frequencies of intra- and intergeneric conjugations.

Recipients	Donors	MIC	MIC of Transconjugants	Transfer Frequencies	*p*	F
E. coli J53	*E. coli (ENT-P1)*	4	4	$(2.16\text{-}4.79) \times 10^{-2}$	0.715	0.376
	E. coli (ENT-W1)	4	4	$(1.38\text{-}3.15) \times 10^{-1}$		
	E. coli (ENT-W3)	8	16	$(1.64\text{-}2.81) \times 10^{-1}$		
K. pneumoniae	*E. coli (ENT-P1)*	4	4	$(5.14\text{-}6.27) \times 10^{-4}$		
	E. coli (ENT-W1)	4	4	$(1.71\text{-}3.95) \times 10^{-3}$		
	E. coli (ENT-W3)	8	4	$(3.91\text{-}4.89) \times 10^{-3}$		

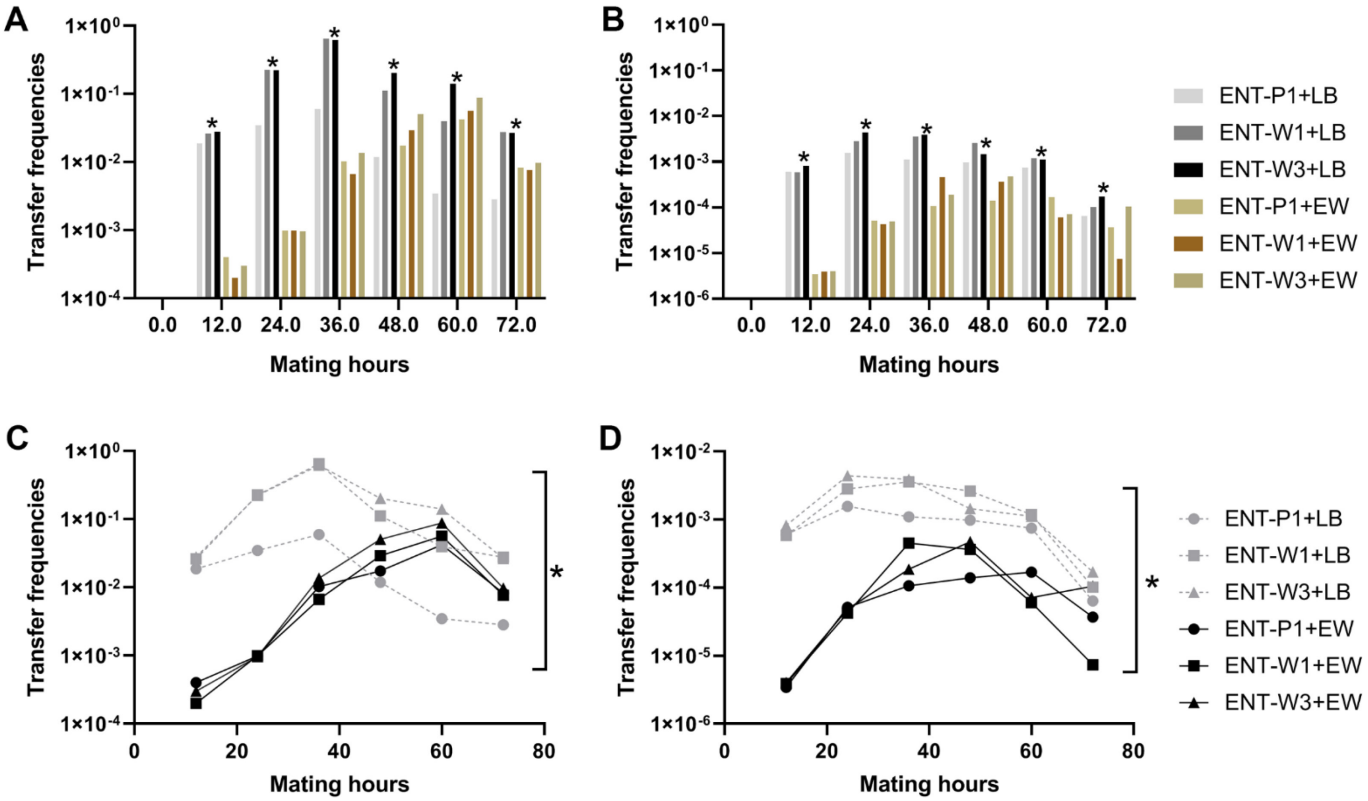
These data were analyzed by one-way ANOVA.

W3). Furthermore, the current results indicated that waterborne *Enterobacteriaceae* isolates resistant to colistin exhibited both high multidrug resistance (6/8) and genetic diversity. Two *mcr-1* positive strains from two water environment sites near the hospital belonged to different sequence types.

Conjugative transferability of mcr-1-gene-carrying strains from different origins

To clarify the pervasive dissemination of colistin resistance, the conjugative transferability of the *mcr-1* gene-carrying strains was evaluated within and across genera. The transfer of colistin resistance was successfully achieved in both intra- and intergeneric transconjugants, as determined by antimicrobial resistance selection. These findings indicate that colistin resistance can be transferred via conjugation within and across genera. A comparison of transfer frequencies revealed that intrageneric transconjugants with *E. coli J53* as the recipient exhibited higher frequencies (range from $(2.16\text{-}4.79) \times 10^{-2}$ to $(1.64\text{-}2.81) \times 10^{-1}$) than intergeneric transconjugants (Table 4). Specifically, donor strains from aquatic environments displayed comparatively higher transfer

Figure 2. The transfer frequencies under LB broth and environmental water conditions over mating periods. **A.** Intrageneric conjugation of three donor strains under two culture conditions; **B.** Intergeneric conjugative transfer of three donor strains under two culture conditions; **C.** Two culture conditions lead to different growth dynamics of transconjugants in intrageneric conjugation and **D.** intergeneric conjugation.



Asterisks indicate statistically significant differences ($p < 0.05$) by Wilcoxon signed rank test. ENT-P1 + LB represented ENT-P1 as the donor strain conjugated under LB broth conditions. ENT-W1 + LB represented ENT-W1 as the donor strain conjugated under LB broth conditions. ENT-W3 + LB represented ENT-W3 as the donor strain conjugated under LB broth conditions. ENT-P1 + EW represented ENT-P1 as the donor strain conjugated under environmental water conditions. ENT-W1 + EW represented ENT-W1 as the donor strain conjugated under environmental water conditions. ENT-W3 + EW represented ENT-W3 as the donor strain conjugated under environmental water conditions.

frequencies than the clinical donor strain in both intra- and intergeneric conjugation experiments (Supplementary Figure 2). These results were in alignment with previous studies that bacterial isolates from water environments exhibit elevated conjugative transfer frequencies. However, no statistically significant differences were observed ($p > 0.05$). The levels of colistin resistance among transconjugants demonstrated that the majority of MICs remained consistent with the donor strains, with the exception of one intrageneric transconjugant from an aquatic donor *E. coli* (ENT-W3), which presented a two-fold increase in MIC (MIC = 16 mg/L) compared to the original donor (Table 4).

The present study implemented a conjugation experiment (B) to assess conjugation processes under environmental water and LB broth conditions. Distinct growth dynamics were identified among the transconjugant populations. The presence of elevated initial nutrient concentrations was found to be conducive to optimal bacterial proliferation. Intrageneric conjugation in nutrient-rich LB broth resulted in increased transfer frequencies as early as 12 hours, with a maximum observed at 36 hours. Conversely, transconjugants under environmental water conditions exhibited a more gradual and moderate increases in transfer frequency, reaching their peak levels at 60 hours (Figure 2). The Wilcoxon signed-rank test yielded a statistically significant difference in transfer frequencies under different culture conditions ($p = 0.0312$). Transfer frequencies within and across genera were consistently higher in nutrient conditions compared to environmental waters ($p = 0.019$). As time progressed, the disparities between the two cultivation conditions waned, particularly with regard to intrageneric conjugation (Figure 2).

Discussion

The rise of multiple drug resistance among *Enterobacteriaceae* severely impacts patients' health. This study explores the distribution and antimicrobial resistance patterns of MDR *Enterobacteriaceae* BSI among patients in this region. The most predominant strains were *E. coli* and *K. pneumoniae* during the study period. These strains exhibited high resistance to ampicillin (86.8%), quinolones (70.4% to 75.5%), and cephalosporins (50.9% to 67.9%). These resistance patterns coincided with those of previous studies [18]. Among the MDR *Enterobacteriaceae* clinical isolates, 35.8% produced ESBLs, while 32.7% were CRE. The advent of CRE in conjunction with MDR presents a substantial therapeutic quandary. Consequently, colistin, designated as the antibiotic of last resort for CRE, assumes paramount importance in these circumstances. Unfortunately, the emergence of widespread colistin resistance has emerged as a pressing public health concern [19–21], underscoring the necessity for concerted response measures. In this study, the colistin resistance rate was 4.4% (7/159) among bloodstream isolates from 159 inpatients. In contrast, a separate clinical study conducted in Zhejiang province reported a colistin resistance rate of 2.8% [22]. A review of the extant literature reveals notable variability in colistin resistance rates, ranging from 0.8% to 20% [15,23,24]. This discrepancy may be attributed to disparities in the populations and sample types examined. Sader *et al.* observed higher resistance rates among isolates from ICU patients compared to those of non-ICU patients. This suggests that ICU admission could increase the risk of colistin resistance [25]. The majority of inpatients enrolled in the present study received critical care, including comprehensive and respiratory-related ICU care (PCCM), which may have contributed to the relatively high resistance observed. Additionally, factors contributing to colistin resistance include prior exposure to antibiotics, previous CRE infection, and consumption of colistin-exposed meat or water sources [26]. Taken together, these findings underscore the necessity for further detailed investigations to clarify transmission routes and develop effective control strategies for colistin resistance.

Despite no discernible differences in characteristics between colistin-susceptible and colistin-non-susceptible patients, a prolonged hospital stay was observed in the latter. Preliminary research in ICU settings has indicated that prolonged hospitalization, specifically in the ICU, is a significant risk factor for colistin resistance [27]. It is crucial to acknowledge that this could potentially lead to the development of colistin-resistant bacteria of nosocomial origin. However, the routine testing for colistin susceptibility remains inadequate in most hospitals. This highlights the need for ongoing surveillance of colistin resistance.

The prevalence of the colistin resistance gene *mcr-1* in clinical isolates has been relatively low [24]. In this study, a *mcr-1*-positive *E. coli* isolate was identified in an ICU patient with no prior exposure to colistin. The positivity rate of the *mcr-1* gene (1/159) is consistent with the findings of other clinical studies. Since its initial identification, the *mcr-1* gene has been widely documented in bacterial isolates derived from animals, food, humans, and the environment. However, the rampant misuse of antibiotics in China's agricultural

sector remains a pressing concern, as this practice exerts selective pressure on resistance genes, including *mcr-1*. Numerous studies have demonstrated that the extensive dissemination of *mcr-1* may have an environmental origin, suggesting that environmental water sources may serve as a potential reservoir [28,29]. A previous study has identified a high abundance of the *mcr-1* gene in the downstream region of the Yangtze River [17]. The Chengnan River is a tributary of the Yangtze River, a body of water with a long history of serving as a source of drinking water and irrigation. Consequently, further investigation was undertaken to determine the presence of the *mcr-1* gene in the water environments surrounding the hospital in the Chengnan River. A total of eight *Enterobacteriaceae* isolates resistant to colistin were obtained from five distinct locations within the river, comprising seven *E. coli* and one *C. freundii*. Two of the eight *E. coli* isolates were found to be *mcr-1* positive, consistent with other studies indicating that *E. coli* plays a significant role as a host for the *mcr-1* gene [30]. Consequently, the hypothesis was formulated that these waterborne *mcr-1*-harbouring-*E. coli* strains were highly suspected as the underlying transmission agent. However, the MLST analysis revealed that the three *mcr-1*-positive *E. coli* belonged to different sequence types (ST10, ST131, and ST155). One potential explanation for this contradiction is that the present study focused exclusively on hospitalized patients with bloodstream infections by MDR *Enterobacteriaceae*. Giani *et al.* reported a high prevalence (38.3%) of *mcr-1*-positive strains in healthy children in the Bolivian Chaco [31], and Wang *et al.* also identified the emergence of *mcr-1* in healthy volunteers [32].

Despite the heterogeneity of sequence types, the three *mcr-1*-positive *E. coli* strains displayed a high degree of consistency in the resistance to ampicillin, third-generation cephalosporins, and aminoglycoside antibiotics. The emergence of MDR bacteria and ARGs in aquatic environments poses a significant threat to public health. Specifically, the propagation of ARGs, such as *mcr-1*, is facilitated through horizontal gene transfer. Furthermore, the presence of oxidants, disinfectants, residual antibiotics, and nanoparticles in aquatic environments exerts selection pressure and promotes conjugative transfer [33]. This study employed the One Health approach to investigate potential connections between aquatic environments and clinical settings in the spread of antimicrobial resistance. The results demonstrated that *mcr-1*-positive strains from aquatic origins (ENT-W1 and ENT-W3) exhibited higher transfer frequencies within and across genera in comparison to the clinical isolate (ENT-P1), which aligns with the observations reported by Pérez-Etayo *et al*. [34]. In addition, the transconjugants exhibited colistin resistance (MIC range: 4-16 mg/L) that was equal to or greater than that of the donor strains (MIC range: 4-8 mg/L). These findings underscore the potential for the propagation and amplification of colistin resistance across bacterial genera in aquatic environments, as evidenced by the ability of *E. coli* and other bacteria from the *Enterobacteriaceae* family to survive and persist for extended periods in such environments [35]. This worrisome situation may potentially exacerbate the already critical issue of colistin resistance.

In natural aquatic ecosystems, bacteria typically thrive in relatively nutrient-deprived conditions, in contrast to the nutrient-rich culture medium. The majority of conjugation experiments were conducted under conditions of nutrient-rich culture medium, which diverge from the growth patterns observed in natural aquatic ecosystems. To accurately simulate bacterial growth and conjugation in natural aquatic conditions, this study innovatively established the conjugation experiments (B) under low temperature, static conditions, and prolonged mating periods in environmental water. Distinct conjugation dynamics were observed: in nutrient-rich LB broth, the availability of nutrients promoted rapid bacterial growth, leading to a sharp increase in transconjugants formation. Conjugative transfer frequencies within 24 hours were observed to be elevated under these conditions in both intra- and intergeneric conjugation. Conversely, conjugation in environmental water exhibited delayed transconjugant emergence and significantly lower initial transfer frequencies. This delayed pattern was attributed to the lower nutrient levels, which resulted in lower bacterial densities and impaired cell-to-cell contact. However, following a 60-hour conjugation period in environmental water, differences in transfer frequencies were observed to be shortened within and across genera. To the best of our knowledge, this is the first study to systematically compare and demonstrate the delayed conjugation kinetics in aquatic environments, providing compelling evidence that aquatic environments are the optimal settings for the acquisition and transmission of colistin resistance. This assertion is substantiated by the higher transfer frequency of aquatic *mcr-1*-positive isolates and the optimal conjugation conditions present in environmental water.

The present study has several limitations. Initially, the presence of *mcr-1*-positive *E. coli* was observed

among inpatients and in the aquatic environment surrounding the hospital. However, the current data and the limited population enrolled do not provide sufficient evidence to demonstrate that environmental water sources serve as a direct transmission route. To effectively illustrate the spread of colistin resistance and the *mcr-1* gene, it is essential to include samples from healthy volunteers, animal products, and the environment. Secondly, while the different sequence types of *mcr-1*-positive *E. coli* were identified, whole-genome sequencing of *mcr-1*-positive isolates was not performed. Further studies are necessary to elucidate the mechanisms of *mcr-1* transmission.

Conclusions

This study describes the prevalence, resistance profiles, genotypes, and conjugative transferability of colistin-resistant *Enterobacteriaceae* from hospitalized patients and the surrounding aquatic environment. The findings indicate that the aquatic environment serves as a potential reservoir, thereby facilitating the proliferation of colistin resistance and the dissemination of MDR bacteria. It is noteworthy that waterborne *mcr-1*-positive *E. coli* strains conferred enhanced conjugative transferability and multidrug resistance. Consequently, coordinated and proactive surveillance is urgently needed to monitor and mitigate the spread of colistin-resistant and MDR bacteria. Further investigation into the transmission mechanisms of colistin resistance is crucial for developing effective public health containment strategies.

Findings to date have shown a low rate of *mcr-1* positivity in clinical samples. However, the prevalence of colistin resistance in patients may be underestimated, as the study exclusively focused on patients with MDR *Enterobacteriaceae* bloodstream infections, and colistin has not yet been widely applied in patients. Given the inherent risks associated with the aquatic environment, routine laboratory monitoring of colistin resistance and the *mcr-1* gene is imperative.

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Conflict of interest

No conflict of interest is declared.

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Annex – Supplementary Items

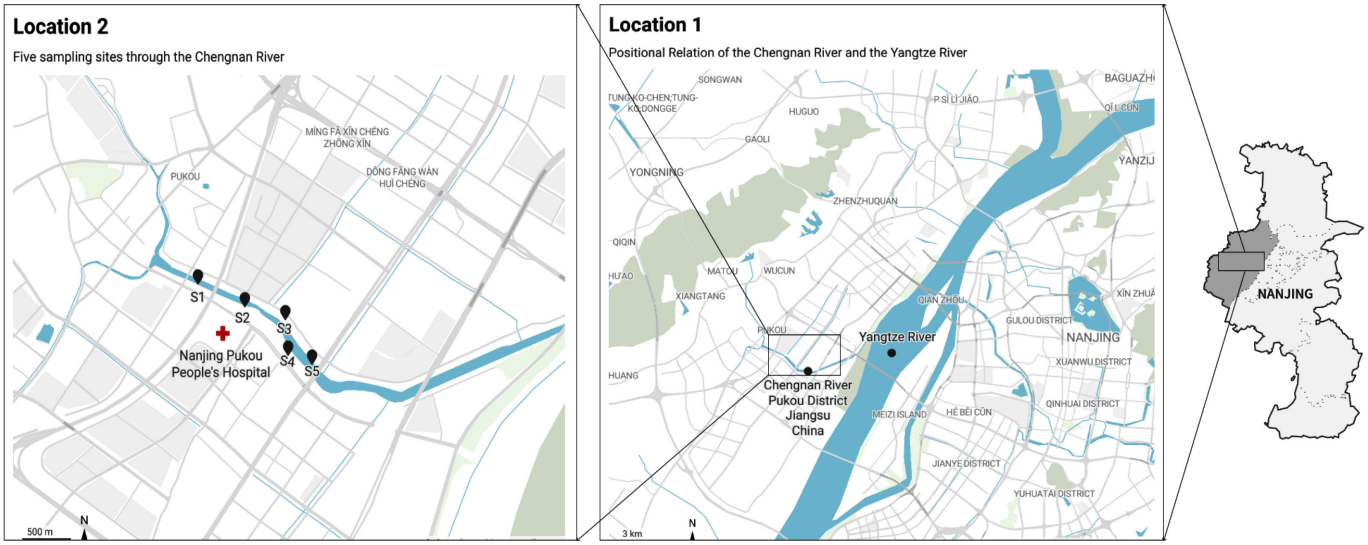
Supplementary Table 1. PCR primers used for amplification of *E. coli* housekeeping genes and *mcr-1* gene.

Genes	Primer sequence (5' → 3')	Amplicon size (bp)	Annealing temperature
adk	F: ATTCTGCTTGGCGCTCCGGG R: CCGTCAACTTTCGCGTATTT	584	54 °C
fumC	F: TCACAGGTCGCCAGCGCTTC R: GTACGCAGCGAAAAAGATTC	469	54 °C
gyrB	F: TCGGCGACACGGATGACGGC R: ATCAGGCCTTCACGCGCATC	460	60 °C
icd	F: ATGGAAAGTAAAGTAGTTGTTCCGGCACA R: GGACGCAGCAGGATCTGTT	518	54 °C
mdh	F: ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG R: TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	452	60 °C
purA	F: CGCGCTGATGAAAGAGATGA R: CATACGGTAAGCCACGCAGA	478	54 °C
recA	F: CGCATTCGCTTTACCCTGACC R: TCTCGATCAGCTTCTCTTTT	510	58 °C
mcr-1q	F: ACACTTATGGCACGGTCTATG R: GCACACCCAAACCAATGAT Probe: GCCCTACAGACCGACCAAGCCG	120	53 °C
mcr-1	F: CAAACCTATCCCATCGCGGA R: AGCTGAACATACACGGCACA	766	59 °C
16SrRNA	F: CCGGGCTCAACCTGGGAA R: TTTAACCTTGCGGCCGTAC Probe: TGCATCTGATACTGGCAAGCTTG	296	59 °C

Supplementary Table 2. Susceptibility profiles of five colistin-heteroresistant strains.

ID	Isolates	MICs of PAP (mg/L)	MICs of colistin in progenies (mg/L)	MICs of colistin heteroresistant strains (mg/L)													
				COL	AM	TZP	ATM	CAZ	FEP	CRO	GM	AK	TOB	CIP	LEV	MEM	IPM
2158	*K. pneumoniae*	4	≤ 2	≤ 2 (S)	≥ 32 (R)	≤ 4 (S)	2 (S)	≥ 64 (R)	2 (S)	≥ 64 (R)	32 (R)	≥ 64 (R)	8 (S)	≥ 1 (R)	≤ 0.5 (S)	≤ 0.25 (S)	≤ 1 (S)
2098	*A. baumanii*	4	4	≤ 2 (S)	≥ 32 (R)	≥ 128 (R)	16 (R)	32 (R)	≥ 32 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)	≥ 16 (R)	≥ 1 (R)	≥ 2 (R)	≥ 4 (R)	≥ 4 (R)
2109	*K. pneumoniae*	8	≤ 2	≤ 2 (S)	≥ 32 (R)	≥ 128 (R)	≥ 64 (R)	32 (R)	≥ 32 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)	≥ 16 (R)	≥ 1 (R)	≥ 2 (R)	≥ 4 (R)	≥ 4 (R)
1903	*K. pneumoniae*	4	≤ 2	≤ 2 (S)	≥ 32 (R)	≤ 4 (S)	2 (S)	16 (R)	≥ 32 (R)	≥ 64 (R)	32 (R)	≥ 64 (R)	≥ 16 (R)	≥ 1 (R)	≥ 2 (R)	≤ 0.25 (S)	≤ 1 (S)
8532	*K. pneumoniae*	16	≤ 2	≤ 2 (S)	≥ 32 (R)	≥ 128 (R)	16 (R)	16 (R)	≥ 32 (R)	≤ 1 (S)	≤ 4 (S)	≤ 8 (S)	≤ 4 (S)	≥ 1 (R)	≥ 2 (R)	≤ 0.25 (S)	≤ 0.25 (S)

Supplementary Figure 1. The geographic distribution of the sampling sites along the Chengnan River in Nanjing.



Supplementary Figure 2. Transfer frequencies of donor strains from different origins in both intra- and intergeneric conjugations.