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Genotypic Detection of Colistin Resistant *Citrobacter freundii* from a Tertiary Care Hospital, Bangladesh

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Abstract

Background: The effectiveness of colistin is threatened by its quick growth in resistance, which is a major global public health concern. Effective surveillance, infection management, and evidence-based treatment decisions are hampered by the absence of epidemiological and genotypic data on colistin-resistant *Citrobacter freundii* in Bangladesh. **Objective:** This study was performed to see the genes accountable for colistin resistance in Citrobacter freundii isolates from different microbiological samples. **Methodology:** In this cross-sectional study, a total of 27 *Citrobacter freundii* isolates were obtained from 500 samples collected between July 2019 and June 2020 using standard culture methods, biochemical, and molecular tests. Their colistin susceptibility was determined using the Minimum Inhibitory Concentration (MIC) agar dilution method. Specific primers were used to detect the colistin-resistant *pmrA*, pmrB, *pmrC*, *phoP*, *phoQ*, *mcr*-1, and *mcr*-2 genes by PCR. **Results:** Amongst the isolated *Citrobacter freundii*, 29.63% were resistant to colistin. Among these resistant bacteria, 50%, 75%, 87.5%, 100%, 12.5% were phoP, phoQ, pmrA, pmrB, and mcr-1, respectively. *pmr*C and *mcr*-2 were not found in any colistin-resistant isolates. **Conclusion:** *Citrobacter freundii* is showing increasing resistance to colistin highlighting the utmost importance for continuous surveillance, rational antibiotic use, and strengthened disease prevention tactics to limit the further spread of colistin resistance.

Keywords: Colistin resistance; chromosomal resistance; enterobacterals; minimum inhibitory concentration; plasmid mediated gene

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Introduction

Globally, antimicrobial resistance (AMR) represents a growing and critical health threat, significantly limiting treatment options for severe bacterial infections. The World Health Organization (WHO) has identified Carbapenem-resistant Gram-negative organisms (CRGNB), particularly carbapenem-resistant Enterobacteriacae (CRE), as critical priority pathogens due to their high mortality and restricted therapeutic choices[1]. The rising prevalence of these organisms, combined with the limited development of novel antimicrobial compounds, has led to renewed reliance on colistin as a last-resort therapy[2,3,4]. However, the widespread clinical use of colistin and its indiscriminate application in animal husbandry have accelerated the emergence of colistin resistance[5,6].

Colistin resistance mainly occurs through chromosomal mutations affecting two-component regulatory systems, resulting in lipid A modification by phosphoethanolamine or 4-amino-4-deoxy-L-arabinose. Additionally, compatible genetic materials (plasmids along with mcr genes) are the chief cause of bacterial resistance to colistin in the infectious world. At least, ten variants of the transferable genes resistant to colistin, mcr 1–10, have been detected[7] with mcr-1 being the most prevalent[8], particularly among *Escherichia coli*, *Klebsiella*

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pneumoniae, and *Salmonella* spp.[9] In Southeast Asia, including Thailand, several mcr variants have been detected in both livestock and human clinical isolates, highlighting the public health threat posed by transferable colistin resistance[10,11].

Citrobacter freundii, a member of the family Enterobacteriaceae, is an opportunistic pathogen attributable to a range of clinical infections, including urinary tract, wound, pulmonary, bloodstream, and central nervous system infections[12,13,14]. Moreover, it serves as an important reservoir of antimicrobial resistance determinants, facilitating horizontal gene transfer[15]. Genomic analyses have identified diverse resistance determinants, including extended-spectrum *β-lactamases* and *AmpC*, plasmid-encoded genes resistant to quinolones, and carbapenemases1[6-24]. In addition, recent reports have documented the occurrence of colistin resistance genes in *Citrobacter* spp. from both clinical as well as animal sources, indicating their emerging role in colistin resistance dissemination[11].

Despite the increasing concern over antimicrobial resistance, data regarding colistin resistance in *C. freundii* in Bangladesh remain inadequate. Hence, this study was executed to find out the frequency and genetic mechanisms of colistin resistance between clinical *C. freundii* isolates in Bangladesh, with the aim of supporting document-based antimicrobial therapy and strengthening infection prevention and control strategies.

Methodology

Study Designs and Population: This cross-sectional study was carried out at Dhaka Medical College Hospital in Dhaka between July 2019 and June 2020. A total of 500 clinical specimens-including wound swabs, pus, sputum, urine, blood, endotracheal aspirates (ETA) and stool-were obtained from inpatients regardless of age and gender, irrespective of prior antibiotic exposure, who were admitted to inpatient wards and intensive care units (ICUs), as well as patients seeking care at the DMCH outpatient department, for microbiological culture and antimicrobial susceptibility testing. Participants declining consent were omitted from the study.

Study Procedure: Every urine, endotracheal aspirate, wound swab, pus and sputum sample materials were added onto blood agar and MacConkey agar and grown under aerobic conditions at 37°C for one day. Blood samples were primarily cultured in trypticase soy broth at optimum time and temperature, followed by subculture onto blood agar and MacConkey agar media. Stool specimens were directly plated onto MacConkey agar. After overnight incubation, culture plates were examined for bacterial growth.

Microbiological detection and characterization of *C. freundii*: C. freundii was identified by Gram staining as Gram-negative bacilli, acidic/alkaline (yellow/red) reaction triple sugar iron (TSI) agar slant. The citrate utilization test was positive. Motile but indole and urease tests negative on motility-indole-urease (MIU) agar. It showed methyl red positive and Voges-Proskauer test negative[25,26]. Detected C. freundii strains were finally confirmed through PCR. Ensured C. freundii isolates were subsequently grown in trypticase soy broth and kept under incubation overnight. The cultures were then centrifuged at 4,000 × g, the top layer was poured off, and the obtained cell deposits were preserved at −20°C up to retrieval of genomic DNA. Detection of resistance-associated genes to colistin was carried out using polymerase chain reaction (PCR) with specific primers.

Colistin insusceptibility testing by MIC agar dilution assay: Colistin insusceptibility was evaluated applying the agar dilution approach for the minimum inhibitory concentration (MIC), following the guidelines set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[27,28]. To categorize isolates as susceptible or resistant, the 2020 EUCAST interpretive criteria were applied while Mueller-Hinton agar served as the testing medium, and Escherichia coli ATCC 25922 was included in respective assay as per reference strain to validate the results.

Preparation of colistin stock solution: By solubilizing 80 mg of colistin base, a stock solution of colistin was prepared, gotten from a commercially available injectable formulation (Forset Pharma Limited), in 10 ml of purified water to achieve a last concentration of 8 mg/ml. The solution was mixed meticulously to ensure comprehensive dissolution before use in subsequent susceptibility assays.

Preparation of Mueller-Hinton agar plate having different concentration of colistin: Mueller-Hinton agar plates having changing concentrations of colistin were assembled by first dispensing 50 mL of sterile Mueller-Hinton agar into each plate. The agar was then added with appropriate amounts of the previously arranged colistin stock solution (8 mg/mL) to achieve last concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 µg/ml. Specifically, 12.5 µl, 25 µl, 50 µl, 100 µl, 200

µl, 400 µl, 800 µl, and 1,600 µl of stock solution were added to the respective plates. The agar was rigorously mixed to confirm even distribution of the antibiotic before solidification [27,28].

Inoculum preparation: A 0.5 McFarland standard (29) was used against the cell suspension turbidity, which corresponds to a bacterial concentration of nearly 1×10^8 CFU/ml. To get a working inoculum of about 1×10^7 CFU/mL, a ten-fold dilution was attained by combining 1 ml of the standardized suspension with 9 ml of sterile normal saline. For agar dilution assays, 1 µL of the diluted inoculum was carefully poured into each Mueller-Hinton agar plate, distributing an estimated 1×10^4 CFU per spot. All inoculated materials were kept at 37°C under aerobic conditions for entire day before assessment.

Inference of MIC: Interpretation of MIC for colistin was done following the 2020 European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Strains having MIC more than 2 µg/ml were regarded as resistant, on the other hand, those with an MIC of 2 µg/ml or less were classified as susceptible.

Identification of colistin-resistant genes by PCR: Conventional PCR was employed to ensure colistin resistance determinants in C. freundii isolates. The specific primer sequences instigated in this study are outlined in Table 1. All PCR assays were performed in the Department of Microbiology, Dhaka Medical College, following standard laboratory protocols to ensure replicability and accuracy.

Table 1: Oligonucleotide sequences of primers used in the identification of colistin-resistant genes in *C. freundii*

Genes	Primer	Sequence	Amplicon	Reference
gnd K1	F	GACGCAGACCGAAATCGAACT	650	30
	R	CGGTTACGGCCAGTGGGAATA		
pho P	F	GAGCGTCAGACTACTATCGA	912	32
	R	GTTTTCCCATCTCGCCAGCA		
pho Q	F	CCACAGGACGTCATCACCA	1594	32
	R	GCAGGTGTCTGACAGGGATT		
pmr A	F	GGTCCAGGTTTCAGTTGCAA	808	32
	R	GCGAAAAGATTGGCAAATCG		
pmr B	F	GCGAAAAGATTGGCAAATCG	659	32
	R	GGAAATGCTGGTGGTCATCTG		
pmr C	F	CTCTCGCCTCGTTCCTGAA	140	32
	R	CGGAGTGGTGTCGAGGATA		
mcr-1	F	AGTCCGTTTGTTCTTGTGGC	320	33
	R	AGATCCTTGGTCTCGGCTTG		
mcr-2	F	CAAGTGTGTTGGTCGCAGTT	715	33
	R	TCTAGCCCGACAAGCATACC		

Genomic DNA Extraction: The bacterial pellets were used for genomic DNA isolates by resuspending them in 300 µL of sterile distilled water, proceeded by thorough mixing and heating vigorously at 100°C for 10 minutes using a thermal bath (DAIHA Scientific, South Korea). The samples later that rapidly chilled on ice and centrifugated at 14,000 × g at 4°C for 6 minutes. The resulting upper liquid portion, containing the DNA, was carefully transferred to fresh microcentrifuge tubes and frozen at −20°C until PCR application[31].

Integration of primer and master mix with DNA template. The PCR assay was executed using 25 µL reaction volume. Each reaction contained 1 µl of 5ˈprimer, 1 µl of 3ˈprimer, 9 µl of pre-mixed reagents (comprising reaction buffer, Taq DNA polymerase, dNTPs, and $MgCl_2$), 2 µl of separated DNA, and 12 µl of nuclease-free purified water. The reaction mixtures were briefly vortexed to ensure homogeneity and then centrifuged prior to thermal cycling.

DNA amplification in thermal cycler: Target DNA sequences were amplified using a thermal cycler (Eppendorf AG, Master cycler Gradient, Hamburg, Germany). The cycling technique comprised of a primary denaturation at 94°C for 10 minutes, subsequently 36 thermal denaturation cycles at 94°C for 1 minute, target-specific annealing at 58°C for 45 seconds, and polymerase extension at 72°C for 2 minutes. The program inferred with a final extension step at 72°C for 10 minutes to confirm complete synthesis of the amplified materials.

Agarose gel electrophoresis assay and examining bands: Agarose gel electrophoresis assay was used to analyze the PCR fragments. Preparation of 1.5% agarose gel (Bethesda Research Laboratories) was done in 1× TBE buffer and run for 30 minutes at 100 V (50 mA). About 5 µl of amplified DNA was incorporated with gel-loading dye in each sample and pipetted onto individual wells, while, for determining fragment sizes, a 100 bp DNA ladder was included in a separate well. Following electrophoresis, the gel was immersed in 0.5 µg/ml ethidium bromide for 30 minutes at ambient temperature and subsequently cleaned in distilled water for 15 minutes. Using a UV transilluminator (Gel Doc, Major Science, Taiwan), DNA bands were visualized, and with digital camera images were captured for documentation.

Statistical Analysis: The data were processed using the SPSS version 22.0. Frequencies and percentages were adopted for nominal variables.

Ethical Clearance: This study was done consistent according to the principles of the Assertion of Helsinki and complied with institutional ethics guidelines. Authorized approval was obtained from the Research Review Committee (RRC) of the Department of Microbiology and the Ethical Review Committee (ERC) of Dhaka Medical College, Dhaka, Bangladesh (Reference: MEU-DMC/ECC/2020/10). All subjects were provided information regarding the study purpose, procedures, and privacy measures, and documented informed consent was gained earlier participation. All data were gathered anonymously and processed using a coding system to preserve the privacy and confidentiality of all participants.

Results

Of the 500 clinical specimens analyzed, 347 (69.4%) were culture positive. Among these, 27 (7.78%) *C. freundii* isolates were recovered from urine, wound swabs, pus, ETA, sputum, stool and blood samples. Specimen-specific distribution is shown in Table 2, with urine yielding the highest proportion (n = 10; 6.89%).

Table 2: Frequency of C. freundii isolated from various sample types

Samples (N)	*C. freundii* (n%)
Urine (N=145)	10 (6.89%)
Wound swab (N=87)	5 (5.75%)
Pus (N=80)	4 (5.00%)
Endotracheal aspirates (N=48)	2 (4.17%)
Sputum (N=50)	2 (4.00%)
Blood (N=52)	2 (3.85%)
Stool (N=38)	2 (5.26%)

*ETA=Endotracheal aspirates, N= Number of different samples, n= number of C. freundii in different samples

Eight colistin-resistant isolates (29.63%) were identified from the 27 C. freundii strains and selected for further study with MICs of 16–256 µg/mL (Table 3).

Table 3: MIC of colistin among the colistin resistant C. freundii by agar dilution method (N=8)

MIC of colistin (µg/ml)	Total number n (%)
≥256	2 (33.3)
128	2 (33.3)
64	1 (16.7)
32	2 (33.3)
16	1 (16.7)
8	0 (0.0)
4	0 (0.0)
≤2	0 (0.0)
Total	**8 (100%)**

PCR screening of the eight colistin-resistant C. freundii isolates showed phoP in 4 (50%), phoQ in 6 (75%), pmrA in 7 (87.5%), pmrB in all 8 (100%), and mcr-1 in 1 (12.5%) isolate. Neither pmrC nor mcr-2 was detected (Table 4).

DNA amplification of the gndk1 gene of C. freundii detected by PCR is shown in Fig. 1.

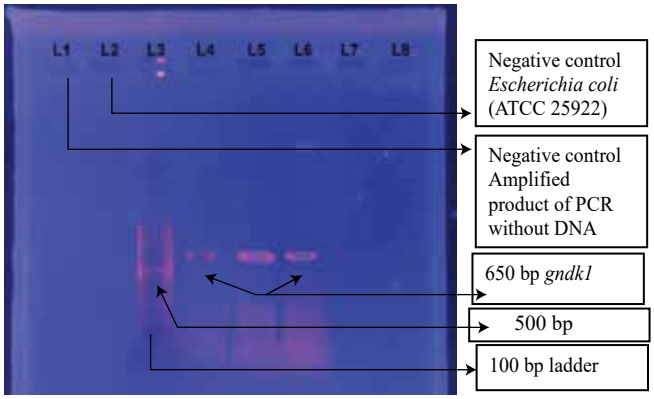

Figure I: Gel electrophoretic image of negative control lacking DNA (lane 1), negative control Escherichia coli ATCC 25922 (lane 2), 100 bp ladder (lane 3), amplified DNA of 650 bp gndk1 gene of C. freundii (lane 4, 5, 6).

Table 4: Prevalence of Colistin Resistance Genes Among Colistin-Resistant C. freundii isolates (n = 8)

Genes	Urine n (%)	Wound Swab n (%)	Pus n (%)	Sputum n (%)	ETA n (%)	Blood n (%)	Stool n (%)	Total n (%)
phoP	2 (25.0)	1 (12.5)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	4(50.0%)
phoQ	1 (12.5)	1 (12.5)	1(12.5)	1(12.5)	0 (0.0	1 (12.5)	1(12.5)	6 (75.0%)
pmrA	3 (37.5)	1 (12.5)	0 (0.0)	0 (0.0)	2 (25.0)	1 (12.5)	0 (0.0)	7(87.5%)
pmrB	2 (25.0)	0 (0.0)	1 (12.5)	1(12.5)	1 (12.5)	2 (25.0)	1(12.5)	8(100.0%)
pmrC	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	0 (0.0)	0 (0.0%)
mcr1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	1(12.5%)
mcr2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0%)

*ETA=Endotracheal aspirates;

DNA amplification of colistin-resistant genes of C. freundii detected by PCR is shown in Fig. 2.

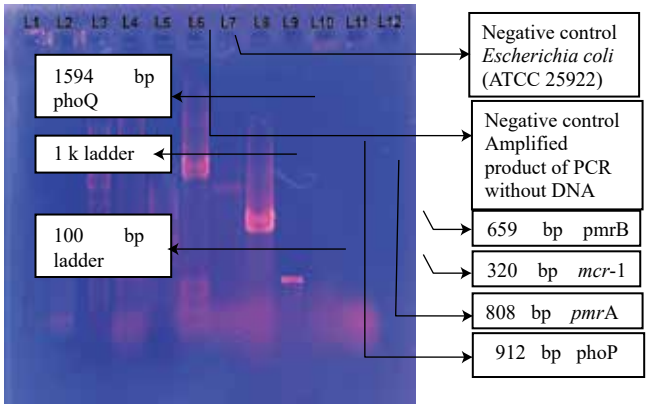

Figure II: Gel electrophoretic image of negative control lacking DNA (TE buffer) (lane 1), negative control Escherichia coli ATCC 25922 (lane 2), 1k ladder (lane 3), amplified DNA of 1594 bp of phoQ gene (lane 4), hundred bp ladder (lane 5), amplified DNA of 912 bp of phoP gene (lane 6), amplified DNA of 808 bp pmrA gene (lane 7), amplified DNA of 659 bp pmrB gene (lane 8), amplified DNA of 320 bp mcr-1 gene (lane 9).

Discussion

Citrobacter freundii has emerged as a prominent nosocomial pathogen, with reported prevalence rates approaching 50.0% cases[34]. The elevated mortality associated with *Citrobacter* infections is likely driven by the widespread implementation of empirical broad-spectrum antibiotic therapy, which exerts substantial selective pressure and accelerates the emergence of multidrug-resistant (MDR) phenotypes. Therapeutic regimens commonly include *β-lactams* particularly extended-spectrum cephalosporins and carbapenems-as well as fluoroquinolones like ciprofloxacin and aminoglycosides[35]. At present, carbapenems remain the cornerstone of therapy for severe *Citrobacterfreundii* bloodstream infections. However, the escalating clinical dependence on carbapenems for the management of diseases caused by gram-negative bacterial pathogens has facilitated the sudden rise and global dissemination of carbapenem resistant *Citrobacter freundii* (CRCF) strains, severely compromising treatment efficacy. This alarming trend necessitates urgent exploration of alternative therapeutic strategies. Consequently, colistin, despite its well-documented nephrotoxicity and neurotoxicity, has re-emerged as a critical last-line agent prescribing for the management of life-threatening infections caused by *Citrobacter freundii* resistant to carbapenem[36].

Various studies have reported that the prevalence of *Citrobacter freundii* ranges from as low as 1.0% to as high as 11.4%[37], which is aligned with our results, where we documented a prevalence rate of 7.78%.

In the present study, *C. freundii* was most frequently isolated from urinary tract infection (UTI) samples, with a prevalence of 6.89%, in agreement with previous reports indicating an increased incidence of *Citrobacter* among urinary pathogens[38,39]. The second highest prevalence was observed in pus and wound swab specimens (5.39%), which is comparable to the findings of Nayar R. However, contrary findings have been reported, with some studies documenting higher isolation rates from pus than from urine samples[37].

Our findings demonstrated colistin resistance in 29.63% of *Citrobacter freundii* clinical isolates, comparable to the 26.7% prevalence reported by Gashaw et al[40]. The MIC values for colistin ranged between 16 µg/mL and ≥256 µg/mL, consistent with previous observations by Hornsey et al[41]. The presence of such high MIC levels is alarming, as it may contribute to inappropriate antibiotic usage and enhance the vertical and horizontal dissemination of resistance determinants among bacterial populations and hosts.

We detected the *phoP*, *phoQ*, *pmrA*, and *pmrB* genes among the colistin-resistant isolates; however, pmrC was not identified which may be attributable to other gene variants. These regulatory determinants mediate lipid A modification of lipopolysaccharides, thereby reducing colistin binding affinity and conferring resistance. The complex regulatory mechanisms underlying colistin resistance may be the reason of coexistence of multiple resistance-associated genes within individual isolates[42].

About 50% and 75% *phoP* and *phoQ* genes, respectively, were positive among the eight colistin-resistant *C. freundii* isolates which is consistent with previous reports[42-44]. In contrast, the prevalence of *pmrA* and *pmrB* was notably higher in our study (87.50% and 100%, respectively) than that recorded in earlier literature, where lower detection rates, including for *pmrC*, have been documented[45]. These differences may imitate distinctions in sampling design, operational approaches, and environmental factors.

Zafer et al[46] and Bir et al[47], who documented *mcr-1* prevalence rates of 5.0% and 6.7%, respectively is matching the present study investigated the prevalence of plasmid-encoded *mcr-1* in only 12.50% of the

isolates, suggesting that colistin resistance is predominantly mediated by chromosomal mechanisms.

In agreement with previous studies, the current study also showed none of the isolates harbored the *mcr-2* gene [46,48-49]. As yet, *mcr-2* has been reported primarily in Belgium. It may be due to limited transmission from animal and environmental reservoirs to human clinical strains, indicating a distinct dissemination pattern compared to *mcr-1*[49].

Though valuable perceptions were accumulated through research but there are some restrictions. First, due to relatively small sample size (n = 8; colistin-resistant *Citrobacter freundii* isolates), precision of prevalence estimates and diversity of colistin resistance determinants within the broader population may not be fully captured. Second, our molecular analysis was restricted to a limited set of resistance-associated genes (*phoP, phoQ, pmrA, pmrB, pmrC, mcr-1*, and *mcr-2*) which may coexist within the same isolates with other important contributors to colistin resistance, including *mgrB, ccrB, ampk36, arnB, arnT*, and additional *mcr* variants etc, were not investigated, and finally, our results restricts the scope of applicability to all other healthcare institutions and the wider national context as it is a single center study.

Conclusion

Colistin-resistant *Citrobacter freundii* with the presence of *pmrA, pmrB, phoP, phoQ,* and *mcr-1* genes determines the increasing threat of antimicrobial resistance and potential treatment failure, while *pmrC* and *mcr-2* genes were not detected in our study. These findings accentuate the importance of incessant surveillance, rational antibiotic use, and effective infection control. Routine monitoring is essential for early detection and strengthening antimicrobial stewardship.

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None

Conflict of Interest
The authors have no conflicts of interest to disclose.

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Authors' contributions
The authors confirm their contribution to the paper as follows: study conception and design: Rahman A, Shamsuzzaman SM. Data collection: Rahman A. Analysis and interpretation of results: Rahman A, Dola NZ, Nabonee MA. Draft manuscript preparation: Rahman A, Dola NZ, Nabonee MA. Supervision: Shamsuzzaman SM. All authors reviewed the results and approved the final version of the manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. All methods were performed in accordance with the relevant guidelines and regulations.

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