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ORIGINAL ARTICLE

Identification of plasmid encoded *qepA* efflux pump gene in *Citrobacter freundii* isolates from a renowned hospital, Bangladesh

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ABSTRACT

Background and Objectives: The emergence of plasmid-mediated resistance to quinolones is a growing global threat that complicates the control of multidrug-resistance (MDR) in *Enterobacteriaceae*. This study was conducted to determine the frequency of the *qepA* gene and its association with antibiotic resistance in *Citrobacter freundii* isolates from various clinical samples.

Materials and Methods: To conduct this study, a total of 27 *C. freundii* isolates collected from urine, endotracheal aspirates, sputum, blood, and stool samples were identified using standard biochemical tests and culture methods. Their susceptibility to ciprofloxacin was determined via the MIC agar dilution method. Specific primers were used to confirm *C. freundii* and detect the *qepA* gene by PCR. Subsequently, DNA sequencing was performed using a capillary method, and the sequences were compared to similar genes available in GenBank.

Results: Among the isolated *C. freundii* strains, 77.78% were resistant to ciprofloxacin. Of these resistant isolates, 9.52% were found to harbor the *qepA* gene. Sequencing and BLAST analysis confirmed a 99% similarity to the *Citrobacter koseri* strain CD4359 plasmid pCD4359 (GenBank accession number KR259132.1) and revealed a point mutation at position 58.

Conclusion: This finding highlights the distribution of the *qepA* gene in clinical isolates, emphasizing the need for continuous molecular surveillance to screen PMQR determinants and to implement effective antimicrobial stewardship strategies.

Keywords: Bangladesh; *Citrobacter freundii*; Efflux pump; Primer; Point mutation

INTRODUCTION

Every year, antibiotic resistance, a major global health challenge, contributes to more than 70,000 deaths worldwide (1). Antibiotic-resistant bacterial infections lead to increased mortality, prolonged treatment duration, slower recovery rates, and higher healthcare costs (2).

Citrobacter freundii, often isolated from the gut of animals and individuals, is a commensal, facultatively anaerobic, rod-shaped Gram-negative bacillus (3). This opportunistic pathogen is responsible for a broad spectrum of infections, including severe diarrhea, urinary tract infections, skin and soft tissue infections, osteomyelitis, and bloodstream infections (4). Prolonged hospitalization or immunosuppressive states are significant risk factors for *C. freundii* infections (5). This pathogen is also a significant cause of nosocomial infection as selective antibiotic pressure of hospital environment facilitates the acquisition of multiple resistance genes, leading to the emergence of MDR strains (6).

Gram-negative as well as Gram-positive bacterial infections are frequently treated with fluoroquinolones (FQs), which are extended-spectrum antimicrobials (7). In Bangladesh, fluoroquinolones are

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frequently prescribed for the treatment of conditions such as gonorrhea, diarrhea, common cold and fever (8). Ciprofloxacin, introduced in 1987, remains one of the most commonly used fluoroquinolones, particularly effective for Gram-negative bacteria such as *Enterobacteriaceae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*, though this drug is less functional for Gram-positive pathogens (9, 10). However, widespread and often indiscriminate use of fluoroquinolones has resulted in increased resistance among bacterial species globally (11).

Several mechanisms such as mutation in target sites, the enzymatic inactivation of drugs, and decreased intracellular accumulation can result in antimicrobial resistance. Among these mechanisms, efflux pumps play a critical role in Gram-negative bacteria since they aggressively expel medicines outside the cell thereby reducing intracellular drug accumulation. These pumps often result in cross-resistance to multiple antibiotics, making them a serious concern in clinical microbiology. The *qepA* gene, which reduces intracellular quinolone accumulation, is a member of the major facilitator superfamily (MSF) and encodes a 14-transmembrane-segment transporter which specifically mediates quinolone efflux (12). Strains expressing *qepA* have been shown to display ciprofloxacin minimum inhibitory concentrations (MICs) 32-64 times higher than those of non-expressing host strains (13).

To date, no study has been carried out regarding *qepA* gene detection in *C. freundii* isolates from human clinical samples in Bangladesh. Therefore, the current study aims to identify and characterize the *qepA* efflux pump gene in isolated *C. freundii*, elucidating its potential contribution to antimicrobial resistance dissemination in this district.

MATERIALS AND METHODS

Study design and eligibility criteria. This cross-sectional study was performed over the course of one year, in the Microbiology Department, Dhaka Medical College (DMC), Bangladesh, from July 2019 to June 2020. Overall, 500 samples, including sputum, blood, pus, endotracheal aspirate, urine, feces, and wound swabs were collected using an aseptic technique and promptly transported to the Microbiology laboratory for further processing. Patients of any sex aged >18 years were included in this study. These encompassed ICU patients who had been on artificial respiratory support for over 48 hours with suspected clinical infections, as well as patients from whom samples were collected for routine culture and sensitivity testing in the Microbiology laboratory. Patients were excluded from this investigation if they were <18 years old, had taken antibiotics within 48 hours before sample collection, or provided samples that were inappropriately labeled or experienced a transport delay of >2 hours. Written consent was obtained from every patient before collecting samples. If patients were too ill, informed consent was obtained from their authorized guardians. Patient confidentiality was maintained throughout the study. This study was approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of the Microbiology Department at Dhaka Medical College (DMC).

Bacterial isolation and identification. Inoculation of each sample of pus, urine, wound swab, sputum and endotracheal aspirate was done onto MacConkey and blood agar media and incubated aerobically for 18-24 hours at 37°C. Primary culture of blood samples was carried out in Trypticase Soy Broth (TSB) at 37°C for 18-24 hours and then subculture was performed on both MacConkey and blood agar media. Only MacConkey agar media was used to inoculate stool samples. After an incubation period of 18-24 hours, the petridishes were inspected for any visible bacterial growth. Biochemical tests, including Citrate utilization, Triple Sugar Iron (TSI), Motility Indole Urease (MIU), and Methyl Red–Voges-Proskauer (MR–VP) tests, were performed to identify *C. freundii*. The detected *C. freundii* strains were confirmed by PCR. For DNA extraction, strains were inoculated into Trypticase Soy Broth (TSB) in microcentrifuge tubes and incubated for 18-24 hours. The tubes were then centrifuged at 4000 × g, the supernatant was discarded, and the pellet was stored at -20°C for DNA separation.

Ciprofloxacin susceptibility testing: Agar dilution method of MIC. The ciprofloxacin susceptibility pattern was determined using the agar dilution MIC method, as mentioned by the Clinical and Laboratory Standards Institute (14). Mueller-Hinton agar was used for antibiotic susceptibility testing, and the CSLI guideline, 2020, were followed to define resistant and susceptible isolates. *Escherichia coli* ATCC 25922 was used as a quality control strain.

Preparation of ciprofloxacin stock solution. As a ciprofloxacin stock solution, an injectable bottle containing 200 mg of ciprofloxacin (Incepta Pharmaceuticals Limited, Dhaka) was utilized; the potency was 200 mg/100 ml, or 2 mg/ml and the molecular formula was $C_{17}H_{18}FN_3O_3$, MW = 331.34 g/mol.

Preparation of Mueller-Hinton agar plate having different concentration of ciprofloxacin. Serial dilutions of ciprofloxacin were prepared in sterile Mueller-Hinton agar to achieve final concentrations of 0.25, 0.50, 1, 2, 4, 8, 16, and 32 µg/ml. Specifically, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µl of stock solution were added to separate 50 ml aliquots of sterilized agar.

Inoculum preparation. The 0.5 McFarland turbidity standard (15) was used to compare the turbidness of the bacterial suspension in normal saline. A ten-fold dilution (one milliliter of test inoculum combined with nine milliliters of normal saline) was performed to achieve approximately 1×10^7 CFU/ml, as the 0.5 McFarland turbidity standard represents approximately 1×10^8 CFU/ml. One microliter of the ten-fold diluted inoculum was added to a Mueller-Hinton agar plate to deliver 10^4 CFU/spot. All inoculated plates were incubated for 18-24 hours at 37°C aerobically.

Inference of MIC. According to CLSI (2020), bacterial strains with a ciprofloxacin MIC of ≥ 1 µg/ml were classified as resistant, and those with an MIC of ≤ 0.25 µg/ml were classified as susceptible.

Identification of *gnd K1* gene of *C. freundii* & *qepA* efflux gene of ciprofloxacin by PCR. The *gnd K1* gene and the ciprofloxacin resistance *qepA* gene of *C. freundii* were detected using conventional polymerase chain reaction (PCR). The primer sequences used in this study are listed in Table 1. The PCR experiments were conducted in the Department of Microbiology at DMC.

Extraction of DNA. An amount of 300 µl sterile water and pelleted bacteria were mixed together, vortexed, and boiled using a heat block (DAIHA Scientific, South Korea) at 100°C for 10 minutes. After rapid cooling on ice, the tube was centrifuged at 14,000 × g and 4°C for 6 minutes. The DNA-containing supernatant was transferred to separate tubes and stored at -20°C for use as a PCR template.

Integration of primer and master mix with DNA template. A 25 µl volume was used as the amplified PCR product. The PCR mixture consisted of 1 mM forward primer, 01 mM reverse primer, 9 mM master mix (containing reaction buffer, Taq polymerase, dNTPs, and $MgCl_2$), 2 µl of extracted DNA, and 12 µl of nuclease-free water in each tube. After brief vortexing, the tubes were centrifuged.

DNA Amplification in thermal cycler. PCR amplification was performed using a thermal cycler (Eppendorf AG, Master Cycler Gradient, Hamburg, Germany). Each run consisted of an initial denaturation at 94°C for 10 minutes; 36 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 45 seconds, and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 10 minutes.

Gel electrophoresis and observation of bands. The resolved products were analyzed by parallel gel electrophoresis using 1.5% agarose (Bethesda Research Laboratories) in 1X TBE buffer at 100 V (50 mA) for 30 minutes, which revealed the PCR bands. Five microliters of amplified DNA were combined with a loading dye and loaded into a separate well on the gel. A 100-base-pair DNA ladder was loaded into another well to estimate the size of the observed bands. The DNA bands were visualized at room temperature after staining with ethidium bromide (0.5 µg/ml) for 30 minutes and destaining with distilled water for 15 minutes. The bands were observed via a UV trans-illuminator (Gel Doc, Major Science, Taiwan), and a digital camera was then used to take a picture.

Table 1. Nucleotide sequences of primers used for identification of *C. freundii* and ciprofloxacin resistant *qepA* gene

Genes	Primer	Sequence	Amplicon	Reference
gnd K1	F	GACGCAGACCGAAATCGAACT	650	16
	R	CGGTTACGGCCAGTGGGAATA		
qep A	F	GCAGGTCCAGCAGGGGTAG	199	6
	R	CTTCCTGCCCGAGTATCGTG		

DNA sequencing procedure. A DNA purification kit (FAVORGEN Biotech Corp.) was used to purify the PCR products for bacterial DNA sequencing. The purified PCR products were sent to 1st BASE Laboratories in Malaysia, where they were sequenced using the capillary technique on an ABI PRISM system.

Statistical analysis. All outcomes were methodically documented. Descriptive analysis was performed using SPSS version 23 and Microsoft Office Excel 2010.

RESULTS

In total, 500 samples were included in this study, of which 347 (69.40%) produced culture-positive results. A total of 27 (7.78%) *C. freundii* isolates were obtained from the 347 culture-positive samples, which included pus, wound swabs, urine, sputum, blood, endotracheal aspirates, and stool. Table 1 displays the prevalence of *C. freundii* isolates from different specimens. The highest number of *C. freundii* isolates was obtained from urine, which accounted for ten cases (6.89%).

Out of 27 isolated *C. freundii*, 21 ciprofloxacin-resistant isolates were selected for further study (Table 2). The MIC range for these 21 ciprofloxacin-resistant *C. freundii* isolates was 4-64 µg/ml, with the highest proportion (42.85%) exhibiting an MIC of 32 µg/ml.

The distribution of the quinolone-resistance *qepA* gene among ciprofloxacin-resistant *C. freundii* isolates, as identified by PCR across various sample types, is displayed in Table 3. Two (9.52%) of the 21 quinolone-resistant isolates were positive for the *qepA* gene.

Table 2. Prevalence of *C. freundii* in different clinical specimens

Samples	*C. freundii* n (%)
Pus & Wound swab (N = 167)	9 (5.39)
Urine (N =145)	10 (6.89)
*ETA (N =48)	2 (4.17)
Blood (N =52)	2 (3.85)
Sputum (N = 50)	2 (4.00)
Stool (N = 38)	2 (5.26)

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

DNA amplification of 650 bp *gndk1* gene of *C. freundii* (Fig. 1) and 199 bp *qepA* efflux pump gene of ciprofloxacin detected by PCR is showed in Fig. 2.

Fig. 3 exhibit the DNA sequencing of *qepA* gene using specific primer and according to Fig. 4, the DNA sequence of the PCR-amplified *qepA* gene from *C. freundii* was 99% identical to that of the *Citrobacter koseri* strain CD4359 plasmid pCD4359 (GenBank accession number KR259132.1). Sanger sequencing revealed a point mutation at position 58 in the *qepA* gene.

DISCUSSION

It is estimated that *C. freundii* is a leading cause of nosocomial infection, with a 50% prevalence rate (17). The high fatality rate of Citrobacter infections may be the outcome of the application of empirical antibiotic therapy which results in increased resistance to frequently used antimicrobial agents. Antibiotics, for example, ß-lactams (extended-spectrum cephalosporins, carbapenems), ciprofloxacin, and aminoglycosides are frequently used to treat Citrobacter infections. The universal increase in fluoroquinolone resistance in the *Enterobacteriaceae* family in recent years has raised serious concerns (18). Moreover, in intrinsic resistance of an organism to fluoroquinolones and other antimicrobial agents, the *qepA* pump is one of the responsible mechanisms (19). This gene can be present on a plasmid as well as alongside other resistance genes, mainly those encoding ESBLs. Decreased susceptibility to fluoroquinolones can also occur because the use of non-quinolone antibiotics can promote the persistence of this gene (20, 21).

In this study, ciprofloxacin resistance was reported in 77.78% of *C. freundii* clinical isolates, aligning closely with the findings of 79.81% by Biez L et al. (22). The range of the MIC against ciprofloxacin was from 4 µg/ml to ≥ 64 µg/ml, supporting the data of Tewawong N et al. These elevated MIC values are a serious concern, as they may encourage the irrational use of antibiotics and facilitate the vertical and horizontal spread of resistant genotypes among hosts (23).

Among 21 ciprofloxacin resistant *C. freundii*, 9.52% of them were positive for *qepA* gene. The prevalence of this gene is quite close to that reported in a study by Chen PL et al. This gene, responsible for reducing the accumulation of quinolones inside bacterial cells,

Table 3. Distribution of ciprofloxacin resistant *C. freundii* in various samples. (N = 21)

Organism	Pus & wound swab	Urine	Sputum	Blood	Endotracheal aspirate	Stool	Total
C. freundii	07 (33.33%)	07 (33.33%)	01 (4.76%)	02 (9.52%)	02 (9.52%)	02 (9.52%)	21 (100.00%)

N= Entire number of quinolone resistant *C. freundii*, n=Number of different samples positive for different quinolone resistance genes.

Table 4. Distribution of quinolone resistance genes between quinolone resistant *C. freundii* detected by PCR in different specimens (N=21)

Genes	Blood n (%)	Wound swab and pus n (%)	Sputum n (%)	Endotracheal aspirate n (%)	Urine n (%)	Stool n (%)	Total n (%)
qepA	0 (0.00)	1 (4.55)	0 (0.00)	0 (0.00)	0 (0.00)	1 (4.55)	2 (9.52)

N= Entire number of quinolone resistant *C. freundii*, n=Number of different samples positive for different quinolone resistance genes.

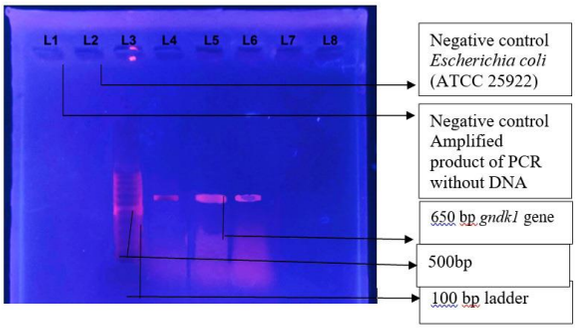

Fig. 1. PCR detection of *gndk1* gene of *C. freundii*. Here Lane 1 is for negative control lacking DNA (TE buffer), lane 2 is for *Escherichia coli* ATCC 25922 negative control, lane 3 shows the 100 bp ladder, lane 4, 5, 6 shows the DNA amplification of 650 bp *gndk1* gene of *C. freundii*.

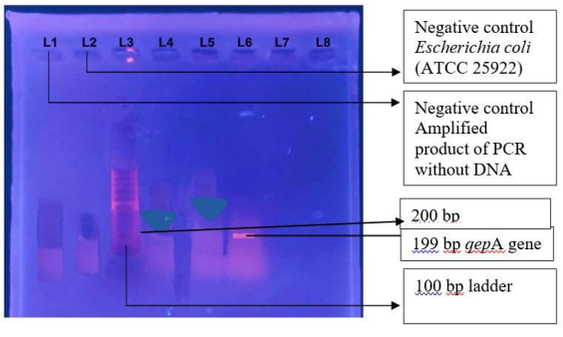

Fig. 2. PCR detection of *qepA* efflux pump gene of ciprofloxacin. Here Lane 1 is for negative control lacking DNA (TE buffer), lane 2 is for *Escherichia coli* ATCC 25922 negative control, lane 3 shows the 100 bp ladder, lane 6 shows DNA amplification of 199 bp *qepA* gene.

CGGGCAGGAAGGAACAGCGCCCCGACCGCCAGCCCGGCCAG
CAGCGCAGCCATCGAGGCGAGGCCCGCTCCATGCTCGGCCAA
CTGCTTGAGCCCGTAGATCGTCAGCAGCACCGCCGCCAGCGA
CAGCAGCACGCTGGCCAGGTCCAGGTGCCCCGCGTCCGGATC
ACGATACTCGGGCAGGAAG

Fig. 3. DNA sequence of amplified PCR product of *qepA* gene using specific primer

has been observed extensively in various Gram-negative species in several Asian as well as African countries. This comparatively low prevalence of the quinolone-resistant gene is mainly due to its restricted host range, which is itself a result of its newness compared to other plasmid-encoded quinolone-resistant genes (24).

The gene was found to occur more frequently in this study than in some other bacterial studies. Kariuki K et al. (25) found 8.5% of isolates were positive for the *qepA* gene in their study, while Pasom et al. (26) documented the absence of this gene in urinary isolates from a hospital in Thailand; a similar absence was reported by Ehwarieme et al. (27).

Conversely, Ogbolu et al. (28) found that 18.7% of the gram-negative bacterial isolates from a hospital in Nigeria had the *qepA* gene. These variations can be attributed to differences in sampling techniques, identification procedures, and geographical location.

Our study showed that the base sequence of the *qepA* gene had 99% similarity to that of *C. koseri*

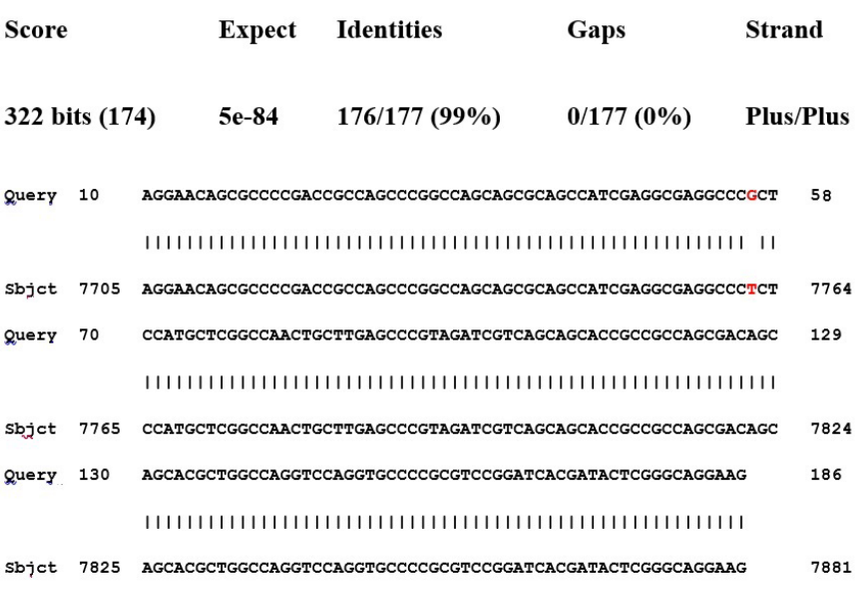

Fig. 4. Sequence similarity between the amplified *qepA* gene and the *qepA* gene from *Citrobacter koseri* strain CD4359 plasmid pCD4359, complete sequence

strain CD4359 plasmid pCD4359 (GenBank accession number: KR259132.1). The *qepA* had a G→C substitution at codon 58 (Fig. 4).

Though our study delivers valuable data, there are several limitations. Our sample size (21 ciprofloxacin-resistant *C. freundii* isolates) is relatively small, which confines the accuracy of prevalence estimations and may not contain the full range of *qepA*-positive plasmids in the population. We focused only on one PMQR gene (*qepA*), whereas other PMQR determinants (*qnr* variants, *aac(6')-Ib-cr*, *oqxAB*) may also cause fluoroquinolone resistance and could remain in the same isolates. Lastly, our study was conducted at a single tertiary hospital, which hinders the generalizability of the findings to other healthcare settings and the wider country.

CONCLUSION

This study indicates that *qepA*-positive *C. freundii* is emerging in humans in Bangladesh. It is important to continuously monitor the commonly used antibiotics to track the growing trend of antibiotic resistance and secure effective treatment outcomes. The horizontal transferability of *qepA* gene highlights the vital need for strict antimicrobial resistance surveillance, prudent fluoroquinolone use, and enforcement of laws prohibiting the sale of over-the-counter antibiotics.

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