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Original Article

Exploring Plasmid-Mediated Quinolone Resistance Gene Diversity among Ciprofloxacin-Resistant Proteus Species in a Tertiary Care Hospital of Bangladesh

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Abstract:

Background: *Increased resistance to fluoroquinolones has been observed in members of the Enterobacteriaceae, including Proteus species. The extensive use of fluoroquinolones and the presence of plasmid-mediated quinolone resistance (PMQR) genes are believed to contribute significantly to fluoroquinolone resistance. This study highlights the prevalence of ciprofloxacin-resistant Proteus species and the presence of specific PMQR genes among them, emphasizing their potential role in quinolone resistance.*

Method: *The study included 30 ciprofloxacin-resistant Proteus isolates collected from wound swab, pus, urine, and blood samples. Identification of Proteus species was performed using culture and biochemical tests. Antibiotic susceptibility test was conducted using the Kirbey-Bauer disc-diffusion method. The minimum inhibitory concentration (MIC) of ciprofloxacin among ciprofloxacin-resistant Proteus species was determined using the agar dilution method. The study aimed to detect specific PMQR genes, namely* aac(6´) Ib-cr, qnrA, qnrB, qnrD, *and* qnrS, *using polymerase chain reaction (PCR).*

Result: *A total of 42 Proteus species were isolated from 310 various samples. Among the Proteus isolates, 71.43% were ciprofloxacin resistant. The study found that 30% of ciprofloxacin-resistant isolates were positive for* aac(6´) Ib-cr *gene, 13.33% for* qnrA *and* qnrS *respectively and 10% for* qnrD *gene. In total, 60% of ciprofloxacin-resistant Proteus isolates were positive for at least one PMQR gene. The* qnrB *gene was not detected among ciprofloxacin-resistant Proteus species. The MIC of ciprofloxacin ranged from 8 µg/ml to 128 µg/ml among ciprofloxacin-resistant Proteus isolates.*

Conclusion: *This study suggests* aac(6´) Ib-cr, qnrA, qnrS, *and* qnrD *genes are emerging in Proteus species, potentially contributing to the development of quinolone resistance and has implications for clinical management and public health.*

Keywords: Proteus, *quinolone resistance, Dhaka Medical College, Bangladesh.*

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Introduction:

Proteus species (spp.), a prominent pathogen found both in healthcare settings and as causative agents of community-acquired infections. *P. mirabilis, P. vulgaris* are particularly noteworthy for their involvement in urinary tract infections, often act as primary infectious agents in patients with long-term indwelling urinary catheters. *P. mirabilis* has been implicated in various clinical scenarios, including bacteremia, empyema, calculi formation, osteomyelitis, and neonatal meningoencephalitis. The diverse modes of transmission of these pathogens allow them to cause infections in different anatomical sites within the human body. Clinical isolation of Proteus species has been reported

from various sources such as abdominal wounds, urine, bladder calculi, epidural ulcers, bronchoalveolar lavage fluid, stool, and infected conjunctiva.[1]

The emergence of multidrug-resistant Proteus spp., coupled with resistance to quinolones, is contributing to an escalating global public health concern.[1] Resistance to quinolones within the Enterobacteriaceae family often arises from chromosomal gene mutations encoding topoisomerase IV, DNA gyrase, regulatory efflux pumps, and porins-related proteins.[2] Plasmid-mediated quinolone resistance (PMQR) has gained significant attention in recent years, encompassing three major mechanisms: protection of quinolone targets via proteins encoded by *qnr* genes, acetylation of ciprofloxacin and norfloxacin by the functional enzyme *aac(62)-Ib-cr,* and involvement of efflux pumps.[3,4] The five known classes of PMQR genes—*qnrA, qnrB, qnrC, qnrD,* and *qnrS*—have been identified in various Enterobacteriaceae, including *Escherichia coli,* Klebsiella spp., Enterobacter spp., Citrobacter spp., Proteus spp., *Serratia marcescens*, and *Providencia stuartii*.[5,6,7]Quinolone resistance rates in clinical isolates of different Gram-negative bacilli have been reported to range from 65% to 70% in India[8,9] and 62.67% in Bangladesh.[10] Limited research has been conducted on the prevalence and distribution of quinolone-resistant Proteus spp. specifically, along with the distribution of various PMQR genes among them. Therefore, this study aims to address this research gap by focusing on ciprofloxacin-resistant Proteus spp. and elucidating the distribution patterns of different PMQR genes within this bacterial group.

Materials and Methods:

Study Design and Sample Collection:

This cross-sectional study was performed in the department of Microbiology of Dhaka Medical College from January to December 2016. Ethical clearance was taken prior to the commencement. A total of 310 clinical specimens, including wound swab, pus, urine, and blood, were collected consecutively from the patients with clinical indications of infection at Dhaka Medical College Hospital.

Identification of Proteus Species:

The specimens cultured both on blood agar media and MacConkey's agar media, incubating at 37ºC for 24-hours in an aerobic environment. The identification of Proteus spp. was based on distinctive swarming growth on blood agar, a distinct fishy odor, and the presence of non-lactose fermenting colonies on MacConkey's agar. Biochemical profiling revealed all Proteus isolates to be non-lactose fermenters, hydrogen sulfide producers, urease positive, oxidase negative, with *P. vulgaris* additionally testing indole positive.

Assessment of Quinolone Resistance:

Resistance to quinolones was evaluated using the Kirby-Bauer disc diffusion method with a 5µg ciprofloxacin disc, interpreting the inhibition zones as per the CLSI[11] guideline. Isolated colonies were emulsified in sterile saline to match the 0.5 McFarland standard for turbidity. This suspension was then spread onto Mueller-Hinton agar and incubated at 37°C for 24 hours. Resistance was inferred when the inhibition zone measured less than 15mm, intermediate resistance at 16-20mm and sensitive at 21mm or greater.

Determination of Ciprofloxacin MIC:[11]

Minimum inhibitory concentration (MIC) of ciprofloxacin was determined among ciprofloxacin-resistant Proteus isolates using the agar dilution method following the CLSI[11] guideline. The CLSI breakpoints were ≤1 mg/ml for sensitivity, 2 mg/ml for intermediate resistance, and ³4 mg/ml for resistance. A 200 mg base of commercially available antibiotic injection vial (Incepta Pharma Ltd, Dhaka) to a concentration of 2mg/ml (200mg/100ml vial) was used for this analysis.

Molecular Detection of Plasmid-Mediated Quinolone Resistance (PMQR) Genes in Proteus spp.:

PCR method was employed to detect *qnrA, qnrB, qnrD, qnrS, and aac(6´)-Ib-cr* PMQR genes among ciprofloxacin-resistant Proteus spp. The sequences and the base pairs of individual primers are mentioned in Table 2.

Bacterial Pellet Preparation:

A loopful of bacterial colony was taken into a falcon tube containing trypticase soy broth and incubated overnight at 37ºc temperature. Then the tubes were centrifuged at 4,000g for 10 minutes and supernatant was discarded. A small amount of sterile trypticase soy broth was added into the falcon tubes with pellets and mixed evenly. In 2-3 microcentrifuge tubes, an equal amount of bacterial suspension was taken and centrifuged at 4,000 rpm for 10 minutes. The supernatant was discarded and the microcentrifuge tube containing bacterial pellets were kept at -20ºc until DNA extraction. Bacterial DNA was extracted by the boiling method.[12] Three hundred microliter of distilled water was added into microcentrifuge tube containing bacterial pellets and vortexed until mixed. The tubes were boiled for 10 minutes in a heat block and placed immediately into ice and kept for 5 minutes. Centrifugation was done at 14,500g for 6 minutes and 10µl supernatant was used for PCR.

Amplification of DNA:

The cycling parameters followed in this study was as follows: initial denaturation at 95°C for 10 minutes, then 30 cycles of denaturation at 95°C for one minute, annealing at 58°c for *qnrA* and *aac(6′)-lb-cr* , 59.1°C for *qnrB*, 57ºC for *qnrD*, 55.6ºC for *qnrS* , extension at 72°C for 0.5 minute, and final extension at 72°C for 10 minutes.[13]

Visualization of Amplified Products:

Amplified DNA was loaded onto a 1.5% agarose gel, electrophoresis was performed, and the gel was stained with 1% ethidium bromide. Visualization occurred under UV light.

Results:

A total of 42 (13.55%) Proteus spp. were successfully isolated from the 310 diverse wound swab, pus, blood, and urine samples. Among them, 32 (76.19%) were identified as *P. mirabilis*, while 10 (23.81%) were classified as *P. vulgaris*. Most of these Proteus isolates exhibited ciprofloxacin resistance, underscoring the gravity of antibiotic resistance challenges. Of the 42 Proteus spp., 30 were resistant to ciprofloxacin, of which 23 (76.67%) being *P. mirabilis* and 7 (23.33%) *P. vulgaris*. The Minimum Inhibitory Concentration (MIC) of ciprofloxacin among these 30-resistant Proteus spp. varied, ranging from 16 µg/ml to ≥256 µg/ml. Notably, a significant proportion 12 (40%) exhibited an MIC of 64 µg/ml indicating a heightened resistance level (Table 1).

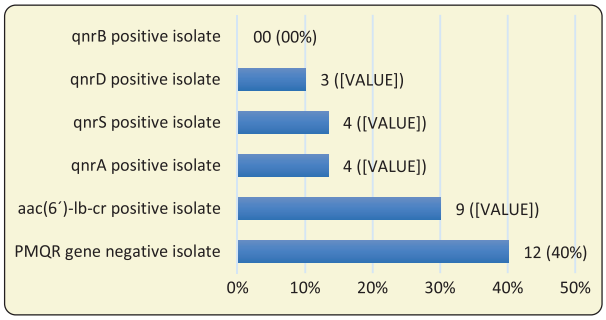
Table 1. Demonstrates MIC of ciprofloxacin in ciprofloxacin resistant *Proteus* spp (N=30).

MIC of ciprofloxacin (µg/ml)	*Proteus* spp. n (%)
≥256	0 (0.00)
128	6 (20.00)
64	12 (40.00)
32	7 (23.33)
16	5 (16.67)
8	0 (0.00)
4	0 (0.00)
2	0 (0.00)
Total	30 (100.00)

Table 2. List of the primer pairs used in this study.

Primer name	Sequence (5´to 3´)	Bp
qnrA	F:CAGCAAGAGGATTTCTCACG R:AATCCGGCAGCACTATTACTC	630
qnrB	F:GGCTGTCAGTTCTATGATCG R:SAKCAACGATGCCTGGTAG	488
qnrD	F:CGAGATCAATTTACGGGGAATA R:AACAAGCTGAAGCGCCTG	581
qnrS	F:AACAAGCTGAAGCGCCTG R:GCAAGTTCATTGAACAGGGT	428
aac(6')-Ib-cr	F:TTGGAAGCGGGGACGGAM R:ACACGGCTGGACCATA	260

Molecular analysis revealed that among the 30 ciprofloxacin-resistant Proteus spp., 9 (30%) were positive for the *aac(6′)–lb-cr* gene, 4 (13.33%) for *qnrA*, 4 (13.33%) for *qnrS*, and 3 (10%) for *qnrD* gene. No isolates were positive for *qnrB* gene. Two Proteus isolates were positive for multiple PMQR gens (Figure 1). One of them was positive for both *qnrA* and *aac(6')-Ib-cr* and the other was positive for both *qnrS* and *aac(6')-Ib-cr* genes. In total, 18 (60%) isolates were positive for Plasmid-Mediated Quinolone Resistance (PMQR) genes, and notably, all positive isolates belonged to the *P. mirabilis*



* Two Proteus isolates were positive for multiple PMQR genes.

Figure 1: *Distribution of PMQR genes in ciprofloxacin resistant Proteus spp.(N=30)*

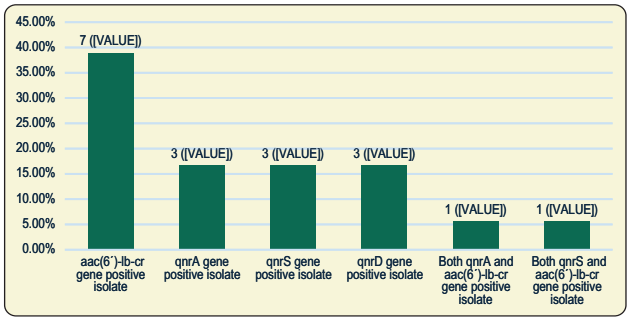


Figure 2: *Distribution of qnrA, qnrB, qnrD , qnrS and aac(6′)-lb-cr genes among the PMQR gene positive P. mirabilis (N=18)*

species. Strikingly, none of the *P. vulgaris* isolates tested positive for PMQR genes, suggesting different resistance mechanisms Figure 2. Representative PCR amplifications of *aac(6′)–lb-cr, qnrA, qnrB, qnrD, and qnrS* genes are visually presented in Figure 3.

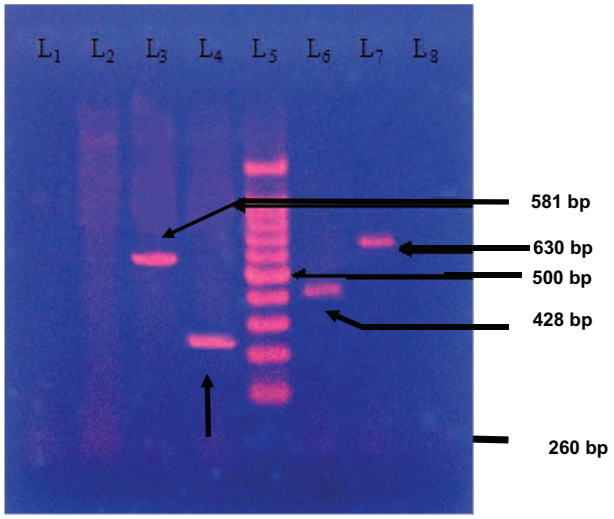


Figure 3: *Photograph of gel electrophoresis of amplified DNA. Negative control without DNA (Lane 1). Negative sample (Lane-2). Amplified DNA of 581 bp for qnrD gene (Lane 3) and amplified DNA of 260 bp aac(6′)-lb-cr (Lane-4).). Hundred bp DNA ladder (Lane 5). Amplified DNA of 428 bp for qnrS gene (Lane 6) and amplified DNA of 630 bp for qnrA (Lane-7). Blank (Lane 8).*

Discussion:

In recent years, the global surge in fluoroquinolone resistance within the Enterobacteriaceae family has become a significant concern.[8,2] Notably, studies have predominantly focused on high rates of fluoroquinolone resistance in *E. coli* and *K. pneumoniae*.[14,15] Limited attention has been given to the prevalence and distribution of quinolone-resistant Proteus spp. and the distribution of Plasmid-Mediated Quinolone Resistance (PMQR) genes among them. In this study, 30 (71.43%) of Proteus spp. exhibited resistant to ciprofloxacin, aligning closely with findings by Pal N (2014) and Bahashwan SA (2013) where 65% and 66.8% Proteus spp. were ciprofloxacin resistant, respectively.[16,17] Multidrug resistance was prevalent among the ciprofloxacin-resistant Proteus isolates, indicating a complex challenge in clinical management. This finding underscores the urgency for innovative therapeutic strategies and heightened surveillance.

PMQR genes were found commonly associated with *E. coli,* Klebsiella spp., and Enterobacter spp. In Proteus *spp.,* Citrobacter *spp.,* Serratia spp. those genes were positive but in negligible amount. The present study uncovered a significant occurrence of *qnrA, qnrB, qnrS, qnrD,* and *aac(6')-Ib-cr* variant genes among ciprofloxacin-resistant Proteus spp., consistent with the studies by Yugendran and Harish (2016) and Jacobey *et al*. (2014) who reported *qnrA, qnrD, qnrS* and *aac(62)-Ib-cr* positive Proteus isolates in their studies.[13,18] This might be a positive finding represents a potential reservoir for the spread of these genes in hospitals and community.

A notable finding of the study was the dominance of the *aac(6')-Ib-cr* gene, with 30% of isolates testing positive. It was close to the study by Yugendran and Harish (2016) where 40% Proteus were positive for *aac(6')-Ib-cr*.[13] This PMQR gene was the most predominant one than any other *qnr* genes in this study and also inconsistent with the results of previous studies, where *aac(6')-Ib-cr* gene was the most widespread PMQR.[19]

In Enterobacteriaceae, the three major groups of *qnr* determinants are *qnrA, qnrB* and *qnrS* and *qnrD* having a minor extent.[20] Four (13.33%) *Proteus* isolates were positive for *qnrA* gene, in this study. Till the present study period, the presence of *qnrA* in *P. mirabilis* exhibits extremely rare.[21] In France, single *qnrA*-producing *Proteus* isolate recovered from 2002 to 2005 and another strain of *P. mirabilis* in 2009.[21,22] *qnrS* gene found positive in 4(13.33%) *Proteus* isolates in present study. Jacobey *et al*. (2014) also reported *qnrS* gene positive *P. mirabilis* in his study.[18] Three (10%) *qnrD* positive *Proteus* isolates were detected in this study. Guillard *et al*. (2014) and Mazzriol *et al*. (2012) also reported *qnrD* positive *Proteus* in their studies, respectively.[23,24]

Single *Proteus* isolates carrying multiple PMQR genes were observed, mirroring findings from prior studies.[25] The co-existence of *qnrA* and *aac(6')-Ib-cr*, as well as *qnrS* and *aac(6')-Ib-cr*, highlights the potential for genetic diversity and complexity in the acquisition of resistance.

Surprisingly, approximately 40% of *Proteus* isolates showed no presence of PMQR quinolone resistance genes. This raises the possibility of other undiscovered *qnr* genes or the involvement of quinolone efflux pumps (*QepA and OqxAB*) in conferring resistance.[26,27]

The observed MIC values for ciprofloxacin among quinolone-resistant Proteus isolates, especially those with positive PMQR genes, were notably elevated.[13] MIC of the quinolone resistant gene positive Proteus isolates were ranging from 16 µg/ml to ≥128g/ml, out of which 40% had MIC of 64g/ml. This finding was in accordance with the study by Yugendran and Harish, (2016) who reported more than 62% quinolone resistant Enterobacteriaceae had MIC

of ≥64μg/ml.[13] High MIC values for ciprofloxacin in this study reflected the extent of treatment problems for these resistant isolates and a need for the continuous evaluation of the commonly used antibiotics.

The horizontal transferability of PMQR genes emphasizes the urgent need for judicious fluoroquinolone use, stringent antimicrobial resistance surveillance, and the enforcement of regulations against over-the-counter antibiotic sales. Continuous evaluation of commonly used antibiotics is imperative to address the evolving landscape of antibiotic resistance and ensure effective therapeutic outcomes.

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