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Original Article

Detection of *qnr* genes and *gyrA* mutation to Quinolone Phenotypic Resistance of UTI pathogens in Bangladesh and the implications

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Abstract

Background: Plasmid-mediated quinolone resistance (PMQR) genes and mutations within the quinolone resistance-determining regions (QRDRs) bequeath to the advent of quinolone-resistant pathogenic microbes. This research was designed to assess the roles of three PMQR genes, *qnrA, qnrB,* and *qnrS,* and any mutation in the *gyrA* gene in the QRDR region as a process of quinolone/fluoroquinolone resistance to urinary tract infection (UTI) bacteria in Bangladesh to guide future management of UTIs. **Methods:** Pathogens from UTIs were isolated and identified, and their phenotype antibiotic susceptibilities were tested for lomefloxacin, ofloxacin, ciprofloxacin, and nalidixic acid. Polymerase chain reaction (PCR) detected the *qnrA, qnrB*, and *qnrS* genes. PCR and sequencing were performed to evaluate any mutation within the QRDRs of the *gyrA* gene. **Results:** Of 100 UTI bacteria, phenotypic resistance was observed in 95.0%, 89.0%, 83.0%, and 71.0% against lomefloxacin, nalidixic acid, ofloxacin, and ciprofloxacin, respectively. PMQR genes: *qnrS, qnrA*, and *qnrB* genes were found in 54.0%, 1.0%, and 4.0% of isolates, respectively. Sequencing the *gyrA* gene revealed single mutation (Ser-83 to Leu) and double mutations (Ser-83 to Leu and Asp-87 to Asn). PMQR genes showed a statistically non-significant association with phenotypic resistance. **Conclusions:** This study confirms the presence of QRDR mutations that were independent of PMQR quinolone resistance genes. Consequently, high resistance against quinolones among uropathogens is evident, and their future use needs to be moderated.

Keywords: Quinolone; PMQR; *qnr*; QRDR; UTI; Bangladesh

Introduction

Fluoroquinolones are among the utmost commonly prescribed antimicrobial medicines because of their towering bioavailability and wide-ranging antimicrobial efficacy (Klein et al., 2018). Fluoro-

quinolones possess excellent oral absorption, extensive distribution in vitro, near to the ground plasma protein binding potential, a comparatively long plasma t½-life, and minimum adverse drug reactions. Consequently, these antimicrobials have emerged as the medicine of choice to treat most Gram-negative bacterial infections (Zhanel et al., 1999, Blandeau, 1999, Mandell and Tillotson, 2002). However, because of their widespread use, including potentially inappropriate use, fluoroquinolones resistance has developed in quite a lot of clinically relevant microbes that comprise *Enterobacteriaceae* (Dalhoff, 2012, de Lastours et al., 2014, Mitra et al., 2019). Plasmid-Mediated Quinolone Resistance (PMQR) and the mutations within the chromosomal quinolone resistance-determining regions (QRDRs) contribute to the development of the quinolone-resistant mechanism of pathogenic microorganisms (Shetty et al., 2019, Tamang et al., 2012). PMQR includes various *qnr* genes, *aac (6')-Ib-cr*, and *qepA*. The *qnr* genes, including *qnrA, qnrB, qnrC, qnrD, qnrE, qnrS, and qnrVC,* encode DNA protection gyrase and topoisomerase IV from quinolone inhibition (Jacoby et al., 2014, Poirel et al., 2012). The first *qnrA* (a PMQR determinant) inactivating quinolone was detected from Birmingham, Alabama, the USA, in 1998 among clinical specimens of *Klebsiella pneumoniae* (Martínez-Martínez et al., 1998).. Multiple studies have subsequently reported that the presence of PMQR genes among *Citrobacter freundii*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *K. pneumoniae*, *Providencia stuartii, Salmonella* spp., *Enterobacter* spp., and *Klebsiella oxytoca* across all continents, including Asia, Europe, Australia, and South America, in recent years (Jonas et al., 2005, Minarini et al., 2008, Nazic et al., 2005, Wang et al., 2003, Ode et al., 2009, Poirel et al., 2005, Rodriguez-Martinez et al., 2006, Nordmann and Poirel, 2005, Cheung et al., 2005).

Both PMQR genes and mutations within the QRDRs contribute to the advent of quinolone-resistant pathogens (Ferrari et al., 2013, Kotb et al., 2019). Gram-negative microbial DNA gyrase is further liable to impeding by quinolones than in topoisomerase IV (Jacoby, 2005). Bacteria evolve resistance by mutations in the QRDR of *gyrA* and *parC* genes, altering the structure of topoisomerase that subsequently reduces the enzyme's affinity to quinolone antibiotics (Moon et al., 2010, Ruiz, 2003, Varughese et al., 2018). The QRDR region lies in the DNA-binding surface of the DNA gyrase enzyme and where amino acid positions 83 and 87 remain the 'hotspots' for mutations for fluoroquinolone resistance (Piddock, 1999). Substitutions of serine-83 (Ser-83) and asparagine-87 (Asp-87) in the *gyrA* gene are among the most repeatedly detected mutations in *Enterobacteriaceae* resistant strains (Varughese et al., 2018). Conversely, the preliminary bull's eye mutations happen more frequently in *parC* in cases of moderately resistant *Staphylococcus aureus* or *Streptococcus pneumoniae*; however, their resistance phenomena increase with additional mutations found in *gyrA* and *parE* genes (Ng et al., 1996, Eliopoulos, 2004, Woodford and Ellington, 2007, Redgrave et al., 2014).

Quinolone and fluoroquinolone include nalidixic acid, ciprofloxacin, ofloxacin, and lomefloxacin. They have been prescribed and consumed to treat urinary tract infections (UTIs) since their availability in the 1970s (Oliphant and Green, 2002, Andersson and MacGowan, 2003). The issue of fluoroquinolones resistance among urinary pathogens not solitarily exists in several low-and-middle-income-countries (LMICs) but equally imposes health threats in high-income-countries (HICs)

(Odoki et al., 2020, Tchesnokova et al., 2019, Banerjee and Anupurba, 2016, de Souza da-Silva et al., 2020, Stapleton et al., 2020, Critchley et al., 2019). Resistance is exacerbated significantly in LMICs by the ease of purchasing antibiotics over-the-counter without a prescription (Bryce et al., 2016, Gravningen et al., 2020, Haque et al., 2019b, Haque et al., 2019a, Godman et al., 2021, Belachew et al., 2021, Jacobs et al., 2019).

The rate of antimicrobial resistance (AMR) has progressively increased internationally. Multiple pieces of research have stated that imprudent prescribing and consumption of antimicrobials are the primary cause of microbial resistance in hospital and community settings (Saleem et al., 2019b, Momanyi et al., 2019, Haque and Godman, 2021a, Saleem et al., 2019a). Multiple earlier research demonstrated that fluoroquinolone resistance remains an independent factor for high rates of mortality and poor clinical outcomes among patients with healthcare-associated infection (HCAIs) (Lautenbach et al., 2010, Haque et al., 2018, Chong et al., 2014, Dalhoff, 2012). Furthermore, the high prevalence of fluoroquinolone resistance raises concerns about whether this group of antimicrobials should be used for prophylaxis (Chong et al., 2014, Terahara and Nishiura, 2019). This is because fluoroquinolone resistance causes difficulties with treating many types of infections, including community-acquired UTIs and HCAIs UTIs, both community and HCAI respiratory infections, cystic fibrosis, chronic obstructive pulmonary disease (COPD), dermatological, intra-abdominal, and sexually transmitted infections, as well as traveler's diarrhea (Davidson et al., 2002, Fuller and Low, 2005, Pletz et al., 2005, Dalhoff, 2012), despite fluoroquinolones becoming resistant to several bacterial pathogens (Xiao et al., 2008, Zou et al., 2003). These are also concerns with the development of fluoroquinolone resistance among TB bacilli (Xu et al., 2009, Takiff and Guerrero, 2011) driven by their imprudent use, i.e., monotherapy or without directly observed therapy (DOTS) (Xu et al., 2009).

In Bangladesh, the self-purchasing of antibiotics is common, enhanced by affordability issues with seeing a physician combined with a culture of self-medication (Darj et al., 2019, Do et al., 2021, Haque et al., 2020), through self-purchasing also exists in many neighboring countries of Bangladesh (Shamsudeen et al., 2018, Mandal et al., 2020, Nepal and Bhatta, 2018, Chautrakarn et al., 2021, Aslam et al., 2020b, Aslam et al., 2020a, Alghadeer, et al., 2018, Faqihi and Sayed, 2021, Gillani et al., 2021, Shrestha et al., 2021). This is apprehension as self-medication with antimicrobials increases their imprudent use and promotes resistance (Ayukekbong et al., 2017, Haque et al., 2019b, Behzadifar et al., 2020, Godman et al., 2021). Bangladesh also provides an appreciable migrant labor force in many realms around the world (Karim et al., 2020). Consequently, increasing the possibility of transmission fluoroquinolones resistant genes among these countries and vice-versa.

We are aware of many ongoing strategies, as well as the blossoming of national action plans (NAP) in Bangladesh, to try and reduce rising antimicrobial resistance (AMR) rates (Haque and Godman, 2021b). As part of these developments, this research was designed to assess the pervasiveness of three PMQR genes, *qnrA, qnrB,* and *qnrS,* and determine mutations in the *gyrA* gene quinolone resistance mechanism amid UTI *Enterobacteriaceae* isolated in Bangladesh. This study further ana-

lyzed the alternation of fluoroquinolone drug affinity and phenotypic susceptibility related to the mutations in either the *gyrA* and PMQR genes. We believe our findings will help direct future strategies as part of the Bangladesh NAP and other approaches to address high AMR rates in Bangladesh.

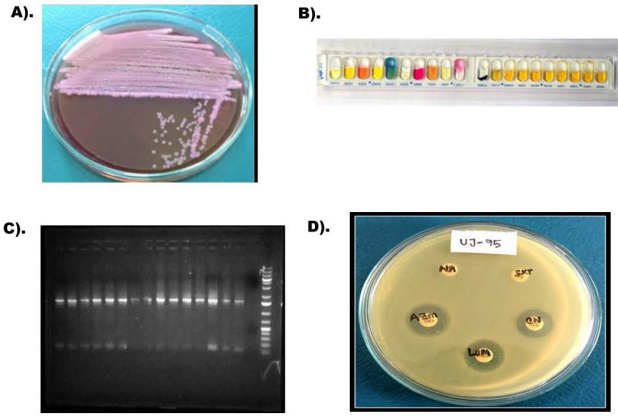
Materials and Methods

Study Design and Specimen Collection

A cross-sectional study was conducted between April 2017 and March 2018 among symptomatic UTI patients attending the outpatient Departments at Gonoshasthaya Samaj Vittik Medical College Hospital, Savar, Dhaka, and Uttara Adhunik Medical College Hospital, Dhaka, Bangladesh. These are privately owned tertiary care teaching hospitals. All the patients who had no history of antibiotic treatment in the preceding 15 days were requested to take part in the study. Those patients who were diagnosed with immunocompromised diseases, different cancers, organ transplants, sexually transmitted infections, and renal disorders were excluded. Midstream clean-catch urine samples were collected from 122 patients who met the study criteria for microbiological investigation. The urine specimens were instantly transported to the laboratory for further examination after collection. Patients were subsequently grouped by gender and age, in groups of ten years, for comparative data analysis.

Bacterial Isolation and Identification

Urine samples were collected in sterile glass tubes and inoculated on a differential culture medium, MacConkey's agar (**Supplementary Figure 1A**), within 2 hours after collection. One loopful of urine was inoculated and incubated at 37°C for 24 hours. After performing quantitative urine cultures, 102 or 103 CFU/mL colony counts were considered to define a probable UTI infection. Colony counts of less than 10^2 CFU/mL were assumed as potentially contaminated. Etiologic proof of identity was confirmed by a rapid biochemical-test kit (API 20E, Biomerieux, Durham, NC) entailing a set of chromogenic panels, carbohydrate batteries, and enzymatic substrates (**Supplementary Figure 1B**) after selecting gram-negative bacterial colony from selective agar plat. Part of the bacterial identities was validated further by amplifying and sequencing the 16S rDNA gene (**Supplementary Figure 1C**) (Van Der Zee et al., 2016). Sequencing services were obtained from a commercial service provider (Macrogen Inc, South Korea). The isolates were conserved in 30% glycerol at - 20ºC in Trypticase Soy Broth (TSB) for further research.



Supplementary Figure 1. Uropathogen Isolation, identification and antibiogram.
A) Urine specimens were inoculated on MacConkey agar medium and incubated overnight at 37°C. Cultural characteristics of a positive-UTI were shown. B) Isolates were identified by API 20E test kits according to the manufacturers' instructions. C) Amplification of of 16s rDNA gene for confirmed identification. D) Phenotypic susceptibility analysis of quinolone antibiotics to the UTI pathogens.

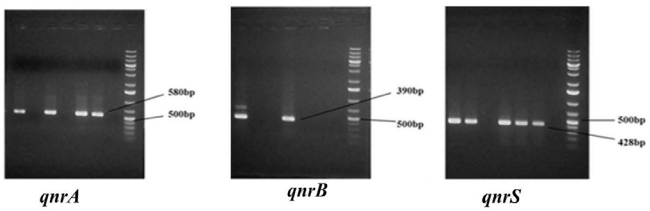
Antimicrobial Susceptibility Testing (AST)

This study analyzed the susceptibility pattern of quinolone and fluoroquinolones separately to each identified UTI species. Phenotypic antimicrobial susceptibilities of the isolates were tested by disc diffusion method (Kirby– Bauer) on Mueller–Hinton agar (Oxoid, Basingstoke, UK) plates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein and Lewis, 2020). Momentarily, a 4-hour bacterial suspension in Mueller–Hinton broth was attuned to a density of McFarland 0.5 equivalent and then squarely streaked on MHA plates to confirm steady growth. The quinolone disc, nalidixic acid (30µg), and the fluoroquinolone discs' ciprofloxacin (5 µg), lomefloxacin (10 µg), and ofloxacin (5µg) were positioned on the bacterial lawn and incubated at 37ºC overnight to evaluate the sensitivity spectrum. Sensitive microbes formed a clear precinct around each disc, and the clear sector diameter was quantified and appraised per CLSI guidelines (**Supplementary Figure 1D**). *Escherichia coli* ATCC 25922 was used as the susceptible-control strains for the AST. In vitro antimicrobial potency of the commonly prescribed quinolone/fluoroquinolone antibiotics, including nalidixic acid, ciprofloxacin, ofloxacin, and lomefloxacin, were tested by the disc diffusion method against the 100 UTI Enterobacteriaceae. Antimicrobial discs were procured from Thermo Fisher Scientific Oxoid Ltd (Basingstoke, England) due to the easy availability of fluproquazones discs.

Detection of PMQR *(qnrA, qnrB, qnrS)* Genes

All the isolates were examined through a polymerase chain reaction (PCR) for genotypic endorsement of plasmid-mediated quinolone resistance genes, including *qnrA, qnrB*, and *qnrS*. Specific pri-

mer sets for the respective genes were designated grounded on previous studies (Cattoir et al., 2007) and synthesized from Integrated DNA Technology (IDT, Singapore). For *qnrA*, the primer pair used was *qnrA-F* 5′-AGAGGATTTCTCACGCCAGG-3′ and *qnrA-R* 5′-TGCCAGGCACAGATCTTGAC-3′with an expected amplicon size 580bp. For *qnrB*, the primer pair used was *qnrB-F* 5′-CCTGAGCGGCACTGAATTTAT-3′ and *qnrB-R* 5′-GTTTGCTGCTCGCCAGTCGA-3′that produced 390 bp amplicon. Primer pair sequences for *qnrS* were *qnrS-F* 5′-GCAAGTTCATTGAACAGGGT-3′ and *qnrS-R* 5′-TCTAAACCGTCGAGTTCGGCG-3′ with its product size 428 bp. For each PCR reaction, bacterial template DNA 2.0 µL was added to a 12 µL of 2X PCR pre-mixture (GeneON, Germany) and five pmol of each primer (1 µL), with deionized water subsequently added to make a final volume of 24 µL. Reactions underwent an initial denaturation at 95 °C for 10 min followed by 32 cycles of amplification (Applied Biosystems 2720 Thermal Cycler, Singapore), consisting of denaturation 30s at 94 °C, annealing 30s at 52-56 °C depending on primer sets, extension 1 min at 72°C, and a final 7 min extension at 72°C. Amplicons were visualized under UV light after electrophoresis through 1.2% agarose gel at 100 volts for 30 minutes. The typical molecular weight marker was run corresponding to quantifying specific amplicon sizes (GeneRuler, ThermoFisher Scientific, MA) (**Supplementary Figure 2**).



Supplementary Figure 2: Detection of plasmid mediated quinolone resistant genes.

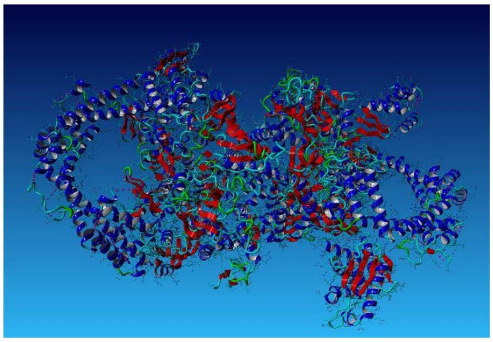
Amplification and Sequence Analysis of QRDR *gyrA* gene

Substitutions of nucleic acids and corresponding amino acids in *gyrA* proteins were studied by PCR amplification followed by sequencing the gene in eight isolates carrying both quinolone-susceptible and quinolone-resistant phenotypes (Oram and Fisher, 1991). The obtained *gyrA* gene sequences of the isolates were compared to other published sequences available in the GenBank database. (http://www.ncbi.nlm.nih.gov). ClustalW Multiple sequence alignment was performed with the highest *gyrA* gene sequence similarity using BioEid software 7.0.

Docking Analyses of Quinolone

Mutations at two specific residues were considered for analysis: Serine83 and Aspartate87. Searching Protein Data Bank (PDB) (Berman et al., 2000), we could find only the structure of *E. coli* gyrase complex bound to inhibitor YacG (PDB ID: 4TMA) (**Supplementary Figure 3**), with no 3D structure of any *Klebsiella* spp. Gyrase complex in PDB format. To predict 3D protein structures *Klebsiella pneumonia,* we retrieved two protein sequences from UniProtKB (Consortium, 2019), namely, *K. pneumoniae* gyrase subunit A (UniProt ID: R4Y7H5) and *K. pneumoniae* gyrase subunit B (UniProt ID:

R4Y6T5). We submitted two protein sequences to the I-TASSER online server (Yang et al., 2015) for protein 3D structure prediction (**Figure 1A**). Mutations at positions 83 (Ser to Leu) and 87 (Asp to Asn) of the amino acid sequences were unified into the normal protein (**Figure 1B**). The structures of two quinolones, ciprofloxacin, and ofloxacin were retrieved in the Structure-Data File (SDF) format from PubChem (Kim et al., 2019) database. These two antibiotics were docked as ligands against the wild-type and mutated gyrase complex receptors. The docking was undertaken in the YASARA platform (Krieger and Vriend, 2014) using the AutoDock VINA (Morris et al., 2009) docking module.



Supplementary Figure 3: *E. coli* gyrase complex bound to inhibitor YacG (PDB ID: 4TMA)

Figure 1.

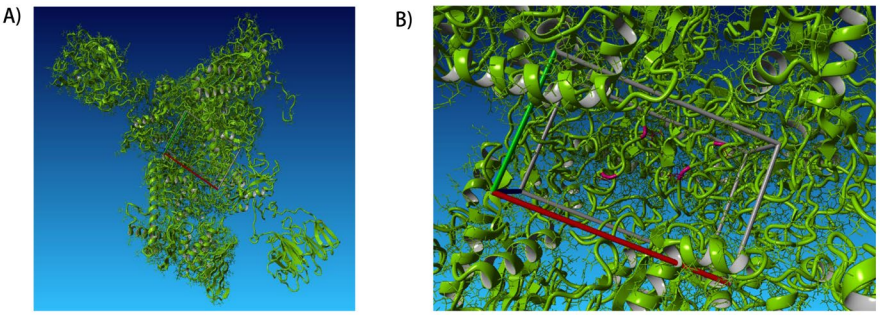


Figure 1: **The predicted 3D structure of *Klebsiella pneumoniae* gyrase complex.**
A). The K. pneumoniae gyrase complex's predicted structure was shown. **B)** Mutated residues in the target site of S83L_D87H mutant K. pneumoniae gyrase were marked in red.

Statistical Analyses

Data were substantiated, key-in spreadsheet scanned, and explored using IBM SPSS statistics data editor (version 21). Missing data were omitted from the bivariate analysis. Descriptive and inferential statistical analyses were conducted to assess the carriage of the three PMQR genes and the *gy-*

rA mutation in UTI pathogens and their phenotypic attributes. Pearson's chi-square test was conducted to examine any association between categorical data, and Yate's correction for continuity was applied where necessary. When the chi-square test's expected frequency cannot be assumed, the Fisher's Exact test results of the 2 x 2 contingency table were reported instead. Two-tailed p-values were calculated to determine statistical significance at the 0.05 level.

Ethical Statement

The Ethics and Research Review Committee of the Jahangirnagar University (JU) Faculty of Biological Sciences approved this study [No. BBEC, JU/M 2017 3(4) dated 15.03.2017]. All the study protocols complied with the Declaration of Helsinki for enrolling human subjects for medical research. Written informed consent was obtained from each adult study patient for collecting their urine samples. Separate written informed consent was taken from parents or legal guardians for patients under 18. Samples were coded to anonymize the study participants' identities and other information.

Results

Urinary Tract Infection Study Patients and Bacterial Etiology

This research analyzed 122 urine samples from symptomatic patients. Of the total samples, 100 UTI bacteria were detected in 100 urine samples, and the remaining 22 samples had no growth, therefore confirmed UTI recovery was in 82.0% of patients (100/122). The recovered 100 UTI isolates with the respective study participants were subsequently analyzed in this study, and 22 subjects were excluded from the next level of analysis. All participants were self-reported symptomatic with either abdominal pain, painful urination, repeated urge to urinate, and an incomplete, void feeling in their bladders. Frequency of the identified UTIs was elevated amid females, 77.0% (n=77) than their male counterpart [23.0% (n=23)].

The age range of the patients was from 8 to 76 years, with those aged between 21-30 years the most vulnerable to UTIs in both genders, with this age group accounting for 35.0% (35/100) of the total number of infections. In each 10-year tier, females had a greater prevalence of UTIs than males. There was no UTI detected among males below 20 years of age, although 18% of females of a similar age range had urinary infections (**Table 1**). However, the higher revealed UTI episodes in females in all the age groups were not statistically significant through the Chi-Squared test (p=0.084). Each urine sample from confirmed cases produced a single UTI pathogen. The most frequently identified UTI bacteria were *Escherichia coli* (n = 28) and *Klebsiella pneumoniae* (n = 44). The other identified bacteria were 11 *Proteus* spp., 10 *Enterobacter* spp., four *Pseudomonas* spp., and three *Staphylococcus* spp.

Table 1: Gender and age-group distributions of urine culture-positive patients (n=100).

Age group (Years)	Male (n=23) Frequency (%)	Female (n=77) Frequency (%)	*p*-value
1-10	0 (0)	4 (100)	0.084*
11-20	0 (0)	14 (100)	
21-30	12 (34.3)	23 (65.7)	
31-40	3 (21.4)	11 (78.6)	
41-50	5 (21.7)	18 (78.3)	
51-60	2 (33.3)	4 (66.7)	
60+	1 (25.0)	3 (75.0)	

*Chi-squared test

Quinolone/Fluoroquinolone Susceptibility Profiling

95.0% of isolates exhibited resistance against lomefloxacin, which indicated the uppermost percentage of resistance midst the quinolones tested. 89.0%, 83.0%, and 71.0% of isolates, respectively, showed resistance to nalidixic acid, ofloxacin, and ciprofloxacin. Ciprofloxacin was the most effective fluoroquinolone among those tested, followed by ofloxacin against UTI pathogens in this study. All four quinolones were found most effective against *Klebsiella pneumoniae,* followed by *E. coli*. They were intermediate effectiveness against *Proteus spp.* and *Enterobacter spp*. UTI pathogen *Pseudomonas spp.* and *Staphylococcus spp.* expressed most resistance in comparison to other bacteria tested (**Figure 2**).

Phenotypic-Genotypic Assessment of PMQR genes

We used PCR to detect three plasmid-mediated quinolone-resistant genes, namely qnrA, qnrB, and qnrS. *qnrS* was the most prevalent plasmid-mediated gene detected in 54.0% of UTI pathogens. However, the other two PMQR genes, *qnrA,* and *qnrB* were detected in 1.0% and 4.0% of pathogens. All the identified *qnrA* and *qnrB* overlay with *qnrS*; consequently, complete co-carriage of two PMQR genes together with *qnrA*+*qnrS* or *qnrB*+*qnrS* was found. One *Escherichia coli* carried all the *qnr* genes while three *Klebsiella pneumoniae* possess *qnrS* and *qnrB.* Intraspecies analyses revealed the highest carriage of PMQR in *Klebsiella pneumoniae* (63.6%), followed by *Enterobacter* spp. (60%), *Proteus* spp. (54.5%) and *E. coli* (46.4%). *Pseudomonas spp.* was found to carry 25% *qnr* genes, but none of *Staphylococcus* spp. carried any of the PMQR genes. (**Table 2**).

Table 2: Identified Plasmid Mediated Quinolone Resistant Genes, *qnrA*, *qnrB*, and *qnrS* in Different UTI Pathogens.

Identified bacteria	Distribution of PMQR[a] genes, frequency (%)[b]
Klebsiella pneumoniae (n=44)	28 (63.6)
Escherichia coli (n=28)	13 (46.4)
Proteus spp. (n=11)	6 (54.5)
Enterobacter spp. (n=10)	6 (60.0)
Pseudomonas spp. (n=4)	1 (25.0)
Staphylococcus spp. (n=3)	0 (0)
Total (N=100)	**54 (54)**

[a] PMQR: plasmid-mediated quinolone-resistant genes, either or all of *qnrA*, *qnrB*, and *qnrS.* [b], row percentage.

As mentioned, we evaluated these UTI pathogens' phenotypic quinolone/fluoroquinolone susceptibilities against nalidixic acid, ciprofloxacin, lomefloxacin, and ofloxacin. Subsequently, we assessed the associations of phenotypic susceptibilities with the carriage of PMQR genes. We did not find any statistically significant correlation with PMQR genes carried by the UTI pathogens with the phenotypic resistance phenomena of either of the four quinolone/fluoroquinolone antibiotics tested (**Table 3**). We further analyzed the association of phenotypic susceptibilities of quinolone/fluoroquinolone with PMQR genes for each UTI pathogen separately, and no statistically significant relation was detected (**Supplementary Table 1**).

Table 3: Association of phenotypic quinolone susceptibilities with plasmid-mediated quinolone-resistant genes.

Quinolone Susceptibility	Presence of PMQR[a] genes (n=100)		p-value [b]
	Yes	No	
Nalidixic acid			
Sensitive	7	4	0.541
Resistance	47	42	
Lomefloxacin			
Sensitive	3	2	1.00
Resistance	51	44	
Ofloxacin			
Sensitive	10	7	0.791
Resistance	44	39	
Ciprofloxacin			
Sensitive	17	12	0.660
Resistance	37	34	

[a] PMQR: plasmid-mediated quinolone-resistant genes, either or all of *qnrA*, *qnrB*, and *qnrS.* [b], the p-value was calculated using Chi-square statistic.

Supplementary Table 1: Dispersal of plasmid-mediated quinolone-resistant genes and phenotypic quinolone susceptibilities of UTI pathogens.

Quinolone resistance trait	Pathogens (n=100)	Phenotypic susceptibility frequency of isolates to four quinolone antibiotics											
		Nalidixic acid			Lomefloxacin			Ofloxacin			Ciprofloxacin		
		S	R	*p* value [b]	S	R	*p* value	S	R	*p* value	S	R	*p* value
Presence of PMQR [a] genes	*Escherichia coli*												
	Yes	2	11	*0.644*	1	12	*0.722*	2	11	*0.572*	4	9	*0.569*
	No	2	13		1	14		3	12		4	11	
	Klebsiella spp.												
	Yes	2	26	*0.463*	2	26	*0.704*	4	24	*0.624*	8	20	*0.557*
	No	2	14		1	15		2	14		5	11	
	Staphylococcus spp.												
	Yes	0	0		0	0		0	0		0	0	
	No	0	3		0	3		1	2		1	2	
	Pseudomonas spp.												
	Yes	0	1		0	1		0	1	*0.750*	1	0	*0.750*
	No	0	3		0	3		1	2		2	1	
	Proteus spp.												
	Yes	0	6		0	6		2	4	*0.273*	1	5	*0.545*
	No	0	5		0	5		0	5		0	5	
	Enterobacter spp.												
	Yes	3	3	*0.167*	0	6		2	4	*0.333*	3	4	*0.167*
	No	0	4		0	4		0	4		0	4	

[a] PMQR: plasmid-mediated quinolone-resistant genes, *qnrA*, *qnrB*, and *qnrS.* [b], the p-value was calculated using Chi-square statistic. S=sensitive; R=resistant.

Mutations Analysis of *gyrA* Gene

Of the total number analyzed, 46% of UTI pathogens did not carry the *qnrA, qnrB*, or *qnrS* PMQR genes. However, 71.7% of these appeared resistant to at least two or more tested quinolones. We subsequently investigated mutations in the hotspot region of the chromosomal *gyrA* gene associated with the exhibited phenotypic quinolone/fluoroquinolone resistance in UTI isolates with and without the three *qnr* genes. *gyrA* gene from three UTI bacteria was amplified and sequenced. Afterward, their translated amino acid sequences were compared regarding the *E. coli* ATCC 25922 strain and R4Y7H5 *Klebsiella pneumoniae* strain. We examined *gyrA* gene sequences of four isolates carrying the PMQR (*qnrS)* gene. Two isolates were *Proteus* spp, *E. coli*, and *Klebsiella pneumoniae*.

No mutation was observed in the *gyrA* region in either of the four isolates (**Figure 3A**). Of the other three isolates, one *E. coli* and one *Klebsiella pneumoniae* manifested double mutations at S83L (substitution of serine to leucine at position 83) and D87N (substitution of aspartic acid to asparagine at position 87). Another *Klebsiella pneumoniae* showed a single mutation at S83L (**Figure 3B**).

Figure 3.

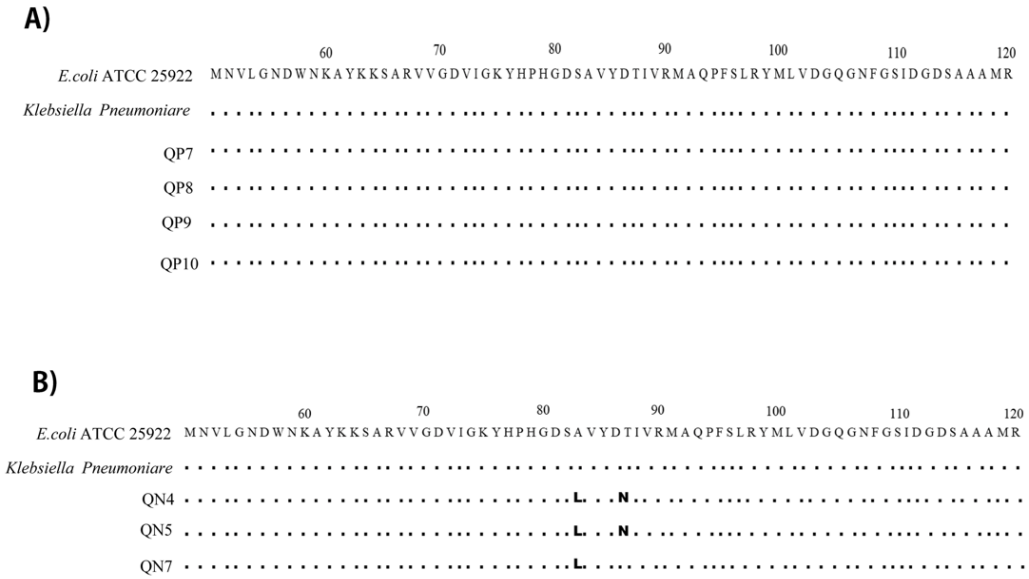


Figure 3: Analyses of mutations in the *gyrA* gene within the quinolone resistance-determining regions (QRDRs).
The amino acid alignment of the *gyrA* gene covering the QRDR region of UTI pathogens, *Klebsiella pneumoniae* and *Escherichia coli*, was related to those of reference *E. coli* ATCC 25922 strain and R4Y7H5 *Klebsiella pneumoniae* strain. Genetic divergence of the QRDR region of the uropathogens was determined by the pair-wise comparison to reference strains. Dots indicate identity, and letters represent substitutions in the UTI pathogens relative to the reference isolates. The findings suggest that *gyrA* sequences of the four bacteria carrying PMQR genes have been aligned, and no mutation was observed (Figure 3A). The amino acid sequences of the *gyrA* gene from the three bacterial isolates without PMQR genes were aligned (Figure 3B). One UTI *E. coli* (QN4) and one *Klebsiella pneumoniae* (QN5) manifested double mutations at S83L (substitution of serine to leucine at position 83) and D87N (substitution of aspartic acid to asparagine at position 87). Another *Klebsiella pneumoniae* (QN7) showed a single mutation at S83L.

Docking results of quinolones with both the reference and mutated *E. coli gyrA* are represented in binding energies. Higher binding energy signifies a more vital interaction amid the ligand and protein. Ciprofloxacin and ofloxacin docking results are portrayed in **Figure 4**. The required binding energy was 8.055 kcal/mol and 8.666 kcal/mol for ciprofloxacin and ofloxacin for the wild-type protein complex, respectively. However, the binding energy became abridged to 6.973 kcal/mol and 7.417 kcal/mol in the case of a mutated protein complex at S83L and D87N (**Figure 4A**).

Similarly, docking results were calculated in the case of *Klebsiella pneumoniae* as well. The binding affinity of ciprofloxacin for wild-type *gyrA* of *Klebsiella pneumoniae* was 7.969 kcal/mol, whereas an affinity for the mutant strain with S83L was 6.528 kcal/mol. The protein binding affinity went down to 6.203 kcal/mol for the strain with two mutations at S83L and D87N (**Figure 4B)**. For ofloxacin, the binding affinity was 7.092 kcal/mol in the case of wild-type protein; however, the affinity reduced to 6.934 kcal/mol and 6.957 kcal/mol for mutated strains with a single mutation at S83L and double mutations at S83L and D87N, respectively (**Figure 4B)**. Docking research revealed that the displacement of quinolone binding sites in a mutated protein complex brings about lower binding energy than the wild one. The reduced affinity could cause the high resistance patterns displayed in this study.

Figure 4.

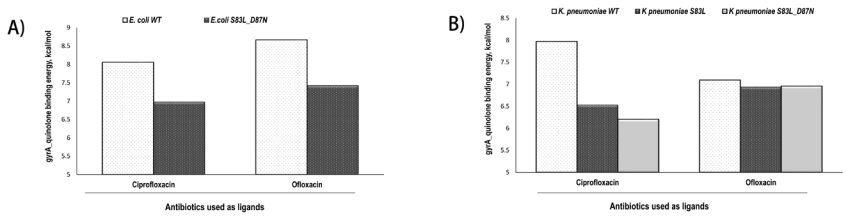


Figure 4: The binding energy of quinolones as ligands for docking into gyrase protein within the quinolone resistance-determining regions (QRDRs).
The comparative binding energy for ciprofloxacin and ofloxacin to wild-type gyrase and mutant-gyrase are shown in Figure 4. Figure 4A depicts the binding energy of ciprofloxacin and ofloxacin for the wild-type gyrase protein complex and double-mutation (S83L and D87N) gyrase proteins of *E. col*. Figure 4B depicts the adhesive strengths of ciprofloxacin and ofloxacin for both single- (S83L) and double-mutation (S83L and D87N) gyrase proteins *Klebsiella pneumoniae* when compared with that wild type. For all cases, binding affinity was detected lower in the case of mutated gyrase proteins.

Discussion

This study investigated phenotypic quinolone/fluoroquinolone susceptibility and carriage of three PMQR genes, namely, *qnrA, qnrB,* and *qnrS,* in urinary tract infecting bacteria in Bangladesh. Further, we analyzed the association of mutations in the QRDR of the *gyrA* gene with the acquisition of quinolone resistance in some selected UTI pathogens.

Our results identified UTI bacteria in Bangladesh exhibiting a high prevalence of phenotypic resistance to four commonly used quinolone antimicrobials. The uropathogens showed higher resistance to lomefloxacin and nalidixic acid than ciprofloxacin and ofloxacin, similar to many other studies conducted in different countries (Colodner et al., 2008, Cao et al., 2011, Santiso et al., 2009, Kim et al., 2020, Lee et al., 2018). Ciprofloxacin was found to be the drug choice to manage patients with UTIs among the four quinolones tested, with similar findings reported earlier in India, Nepal, Bangladesh, and Sri Lanka, as well as many other LMICs (Sedighi et al., 2015, Saksena et al., 2018, Singh et al., 2019, Britto et al., 2018, Hooda et al., 2019).

This study established a high abundance (54.0%) of PMQR genes dominated by *qnrS* in quinolone-resistant urinary *Enterobacteriaceae*, comparable to the earlier studies (Kim et al., 2009a, Poirel et al., 2006). The lower detection of *qnrB* (4.0%) and *qnrA* (1.0%) in clinical isolates were also consistent with other studies (Abd El Salam et al., 2020, Poirel et al., 2006). Despite the presence of different PMQR genes, our study did not find a statistically significant relation between detected *qnr* genes and corresponding phenotyping quinolone/fluoroquinolone resistance.

A significant portion of UTI isolates without bearing *qnr* genes exhibited phenotypic resistance to the same sets of quinolone/fluoroquinolone antimicrobials. The inconsistency of the genotype-phenotype association could be explained by other PMQR genes, such as *aac(6')-Ib-cr, qepA, qnrC, qnrD, qnrE,* and *qnrVC,* that were not investigated in this study (Jacoby et al., 2015, Strahilevitz et al., 2009).

Further, this study characterized the *gyrA* gene mutation mediated quinolone-resistant mechanisms in circulating UTI pathogens of Bangladesh. We found one *E. coli* and *Klebsiella pneumoniae* with two substitutions (S83L and D87N) and one *Klebsiella pneumoniae* with one mutation (S83L) in the *gyrA* gene. Similar mutations were reported in some diarrheal *enterotoxigenic E. coli* (ETEC) in Bangladesh (Begum et al., 2016) as well as UTI pathogens from other countries (Betitra et al., 2014, Lindgren et al., 2003, Varughese et al., 2018). In our study, these three strains were resistant to all tested quinolones without harboring PMQR genes. These results make available further evidence that chromosomal QRDR mutations in sequences encoding *gyrA* perform an indispensable role in quinolone resistance (Moon et al., 2010). The findings also suggest that S83L and D87N mutations in *gyrA* can hinder the broad-spectrum antibacterial activities of quinolones by restricting the DNA gyrase and topoisomerase IV activities (Piddock, 1999). Moreover, docking results of quinolones with wild type and mutated *gyrA* protein from both *E.coli* and *Klebsiella pneumoniae* provided the principle of QRDR mutation-based quinolone resistance (Ruiz, 2003). Mutated *gyrA* protein showed reduced ligand binding energy for both uropathogens, as observed in previous research reports (Varughese et al., 2018, Chu et al., 2020).

This high frequency of quinolone-resistant urinary pathogens is a concern, as quinolones are still the antimicrobials of choice for managing UTIs in Bangladesh and abroad. However, the excessive use of either oral or parenteral quinolones for UTIs and other infections in recent years may enhance high rates of AMR (Holmes et al., 2016). The increased resistance in any currently widely used antibiotic makes treatment decisions difficult. It imposed higher medical expenditure when primary recommended antibiotics do not produce the desired results and/ or alternative antibiotics are prescribed (Strahilevitz et al., 2009). This study showed that *Klebsiella pneumoniae and E.coli* were the most typical pathogen causing complicated and uncomplicated UTIs, which is similar to other studies (Urmi et al., 2020, Founou et al., 2017, Hofer, 2019, Haque and Godman, 2021b). Several different bacteria identified known to cause UTIs, including *Pseudomonas aeruginosa*, *Staphylococcus spp.*, *Proteus spp.*, and *Enterobacter spp.,* have been stated in earlier research reports (Linhares et al., 2013, Urmi et al., 2019). These findings can help to provide empiric guidance on the management of UTIs in Bangladesh and the wider prospect around the globe.

This study also identified urinary infections more commonly in females than males, with a 10-year stratified age grouping revealing a higher prevalence of UTIs among females in all the age classes. Reproductively active women aged 20-39 years accounted for most UTI presentations, similar to others (Urmi et al., 2020, Ara et al., 2021, Smith et al., 2018, Moran et al., 2020). We believe these combined research findings can be used to develop preventive strategies for managing recurrent UTIs among the general population in Bangladesh, especially among women, and we will be following this up.

We are aware of several limitations with this study. Firstly, this study was conducted under a cross-sectional design, and we are aware of the importance of perspectives regarding cross-sectional research. The purposive sampling was also only undertaken in a single community and urban hospital in Bangladesh. Thirdly, risk behavior data among the participants, including the prescribing physicians, were not studied in detail. Fourthly, we could not recruit cases from the initial stages of UTIs of the research subjects as we were typically dealing with recurrent UTIs. In Bangladesh, patients usually seek medical care when the disease process is more advanced. Fifthly, plasmid-mediated quinolone resistance genes including *aac (6')-Ib-cr, qepA, qnrC, qnrD, qnrE,* and *qnrVC,* were not investigated. However, we maintained the internal validity of our results by repeating independent experiments where necessary enhancing the robustness of our findings.

Conclusions

Uropathogens circulating in Bangladesh are highly resistant to quinolone antibiotics. Ciprofloxacin was the most effective fluoroquinolone against tested UTI pathogens, while lomefloxacin appeared the least effective. Acquisition of the *qnrS, qnrA*, and *qnrB* genes carry the spurious association of quinolone resistance in UTI pathogens. However, our findings have disclosed shortcomings of molecular methods of identifying AMR in Bangladesh. The discordance of genotype and phenotype resistance necessitates further studies to ensure precision diagnosis, careful selection of antimicrobials, and rational therapeutic decisions to reduce future AMR rates. Possession of mutation in the QRDR confers quinolone-resistance in uropathogens independently. The findings suggest urgent surveillance and national and global antimicrobial stewardship interventions as part of the NAP in Bangladesh to guide future management.

This study's initiatives and protocols can design further point prevalence surveys (PPS) from more sentinel sites to collect data on resistance and usage of quinolones and other antibiotics. Similar PPS studies can provide a relatively quick assessment of AMR or antimicrobial uses (AMU) in low-resource settings where continuous surveillance is challenging to enhance future care. Notably, the protocols developed in this study can be applied to establish and maintain surveillance systems to collect and use data on AMR and antimicrobial use in hospitals and communities where most antimicrobials are used, and unbiased AMR rates are unknown chiefly.

Article Highlights

- UTI is endemic in Bangladesh, and *Klebsiella pneumoniae* and *Escherichia coli* are the predominant pathologies.
- The uropathogens exhibited high resistance to ciprofloxacin, ofloxacin, lomefloxacin and nalidixic acid.
- Ciprofloxacin currently appears comparatively more effective antimicrobial against urinary pathogens than other tested quinolones
- PMQR gene, *qnrS,* is highly prevalent in Bangladeshi UTI microbes. *qnrA* and *qnrB* genes are also detected.
- UTI bacteria carrying a single mutation (Ser-83 to Leu) and two double mutations (Ser-83 to Leu and Asp-87 to Asn) at the QRDR region of the *gyrA* gene exhibited quinolone resistance.
- Molecular docking analysis also revealed that *gyrA* mutations at Ser-83 and Asp-83 play a critical role in the acquired quinolone resistance.

Author Contributions

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Consent for Publication

All authors reviewed and approved the final version and have agreed to be accountable for all aspects of the work, including any issues related to accuracy or integrity

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Disclosure

The authors declare that they do not have any monetary connection or relationships with any organization, association, or entity directly or indirectly with the subject matter or materials presented in this article. This includes honoraria, expert testimony, employment, ownership of stocks or options, patents or grants received or pending, or royalties.

Data Sharing

The data that support the findings of this study are available from the corresponding author, SI, upon reasonable request

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