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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-resistant (PMQR) genes and mutations within the quinolone resistance-determining regions (QRDRs) resulted in 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 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 nonsignificant association with phenotypic resistance.

Conclusions: This study confirms the presence of QRDR mutations that were independent of PMQR genes. Consequently, high resistance against quinolones among uropathogens is evident, and their future use needs to be moderated.

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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 plas-ma protein binding potential, a comparatively long plasma t½-life, and minimum adverse drug reac-tions. Consequently, these antimicrobials have emerged as the medicine of choice to treat most Gram-negative bacterial infections (Blandeau, 1999; Mandell and Tillotson, 2002; Zhanel *et al*., 1999). However, because of their widespread use, including potentially inappropriate use, fluoro-quinolones 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 in Birmingham, Alabama, 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 sa-kazakii, K. pneumoniae, Providencia stuartii, Salmonella spp., Enterobacter spp., and Klebsiella oxytoca across all continents, including Asia, Europe, Australia, and South America, in recent years (Cheung *et al*., 2005; Jonas *et al*., 2005; Minarini *et al*., 2008; Nazic *et al*., 2005; Nordmann and Poirel, 2005; Ode *et al*., 2009; Poirel *et al*., 2005; Rodriguez-Martinez *et al*., 2006; Wang *et al*., 2003).

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 re-sistance 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 lies on the DNA-binding surface of the DNA gyrase en-zyme 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 (Eliopoulos, 2004; Ng *et al*., 1996; Redgrave *et al*., 2014; Woodford and Ellington, 2007).

Quinolone and the fluoroquinolones 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 (Andersson and MacGowan, 2003; Oliphant and Green, 2002). The issue of fluoro-quinolones resistance among urinary pathogens not solitarily exists in several low- and middle-income countries (LMICs) but equally imposes health threats in high-income countries (Banerjee and Anupurba, 2016; Critchley *et al*., 2019; de Souza da-Silva *et al*., 2020; Odoki *et al*., 2020; Stapleton *et al*., 2020; Tchesnokova *et al*., 2019). Resistance is exacerbated significantly in LMICs by the ease of purchasing antibiotics over the counter without a prescription (Belachew *et al*., 2021; Bryce *et al*., 2016; Godman *et al*., 2021; Gravningen *et al*., 2020; Haque *et al*., 2019a, 2019b; 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 (Haque and Godman, 2021a; Momanyi *et al*., 2019; Saleem *et al*., 2019a, 2019b). 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) (Chong *et al*., 2014; Dalhoff, 2012; Haque *et al*., 2018; Lautenbach *et al*., 2010). 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, dermatological, intra-abdominal, and sexually transmitted infections, as well as traveler's diarrhea (Dalhoff, 2012; Davidson *et al*., 2002; Fuller and Low, 2005; Pletz *et al*., 2005). This may be because the fluoroquinolones have become 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 (Takiff and Guerrero, 2011; Xu *et al*., 2009) driven by their imprudent use, i.e., monotherapy or without directly observed therapy (DOTS) (Xu *et al*., 2009).

In Bangladesh, self-purchasing of antibiotics is common, enhanced by affordability issues with see-ing a physician combined with a culture of self-medication (Darj *et al*., 2019; Do *et al*., 2021; Haque *et al*., 2020); it also exists in many neighboring countries of Bangladesh (Alghadeer *et al*., 2018; Aslam *et al*., 2020a, 2020b; Chautrakarn *et al*., 2021; Faqihi and Sayed, 2021; Gillani *et al*., 2021; Mandal *et al*., 2020; Nepal and Bhatta, 2018; Shamsudeen *et al*., 2018; Shrestha *et al*., 2021). This is apprehension as self-medication with antimicrobials increases their imprudent use and promotes re-sistance (Ayukekbong *et al*., 2017; Behzadifar *et al*., 2020; Godman *et al*., 2021; Haque *et al*., 2019b). 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 the many ongoing strategies, as well as the blossoming of national action plans (NAP) in Bangladesh, to try and reduce the rising 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 analyzed the alternation of fluoroquinolone drug affinity and phenotypic susceptibility related to the mutations in either gyrA or 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 trans-mitted infections, and renal disorders were excluded. Midstream clean catch urine samples were col-lected from 122 patients who met the study criteria for microbiological investigation. The urine spec-imens were instantly transported to the laboratory for further examination after collection. Patients were subsequently grouped by gender and age, in groups of 10 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 agar (Supplementary Fig. 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 102 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 chro-mogenic panels, carbohydrate batteries, and enzymatic substrates (Supplementary Fig. 1B) after se-lecting the Gram-negative bacterial colony from the elective agar plat. Part of the bacterial identities was validated further by amplifying and sequencing the 16S rDNA gene (Supplementary Fig. 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 −20°C in trypticase soy broth for further research.

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 the disk 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 the Mueller–Hinton broth was attuned to a den-sity of 0.5 McFarland equivalent and then squarely streaked on MHA plates to confirm steady growth. The quinolone disk, nalidixic acid (30 µg), and the fluoroquinolone disks, ciprofloxacin (5 µg), lomefloxacin (10 µg), and ofloxacin (5 µg), were positioned on the bacterial lawn and incubated at 37°C overnight to evaluate the sensitivity spectrum. Sensitive microbes formed a clear precinct around each disk, and the clear sector diameter was quantified and appraised per CLSI guidelines (Supplementary Fig. 1D). Escherichia coli ATCC 25922 was used as the susceptible control strain 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 disk diffusion method against the 100 UTI Enterobacteriaceae. Antimicrobial disks were procured from Thermo Fisher Scientific Oxoid Ltd (Basingstoke, England) due to the easy availability of fluproquazones disks.

Detection of PMQR (*qnrA, qnrB, and qnrS*) genes

All the isolates were examined through a polymerase chain reaction (PCR) for genotypic endorsement of PMQR genes, including *qnrA*, *qnrB*, and *qnrS*. Specific primer 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 of 580 bp. For *qnrB*, the primer pair used was *qnrB-F* 5′-CCTGAGCGGCACTGAATTTAT-3′ and *qnrB-R* 5′-GTTTGCTGCTCGCCAGTCGA-3′ that produced a 390 bp amplicon. Primer pair sequences for *qnrS* were *qnrS-F* 5′-GCAAGTTCATTGAACAGGGT-3′ and *qnrS-R* 5′-TCTAAACCGTCGAGTTCGGCG-3′ with its product size of 428 bp. For each PCR reaction, the bacterial template DNA 2.0 µl was added to 12 µl of a 2X PCR pre-mixture (GeneON, Germany) and 5 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 minutes, followed by 32 cycles of amplification (Applied Biosystems 2720 Thermal Cycler, Singapore), consisting of denaturation for 30 seconds at 94°C; annealing for 30 seconds at 52°C–56°C, depending on the primer sets; extension 1 minutes at 72°C; and a final 7-minute 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 Fig. 2).

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 carried out 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: serine-83 and aspartate-87. Search-ing the 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 Fig. 3), with no 3D structure of any Klebsiella spp. gyrase complex in PDB format. To predict 3D protein structures of Klebsiella pneumonia, we retrieved two protein sequences from UniProtKB (UniProt 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 (Fig. 1A). Mutations at positions 83 (Ser to Leu) and 87 (Asp to Asn) of the amino acid sequences were unified into the normal protein (Fig. 1B). The structures of two quinolones, ciprofloxacin and ofloxacin, were retrieved in the struc-ture data file 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.

Statistical analyses

Data were substantiated; the key-in spreadsheet was scanned and explored using IBM SPSS statistics data editor (version 21). Missing data were omitted from the bivariate analysis. Descriptive and in-ferential statistical analyses were conducted to assess the carriage of the three PMQR genes and the gyrA mutation in UTI pathogens and their phenotypic attributes. Pearson's chi-square test was con-ducted 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, Fisher's Exact test results of the 2 × 2 contingency table were reported instead. Two-tailed p-values were cal-culated to determine statistical significance at the 0.05 level.

Ethical statement

The Ethics and Research Review Committee of the Jahangirnagar University (JU), Faculty of Bio-logical 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 re-search. 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 pa-tients under 18 years. Samples were coded to anonymize the study participants' identities and other information.

RESULTS

UTI 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 the 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 and 30 years the most vulnerable to UTIs in both genders and accounting for 35.0% (35/100) of the total number of infections. In each 10-year tier, females had a greater

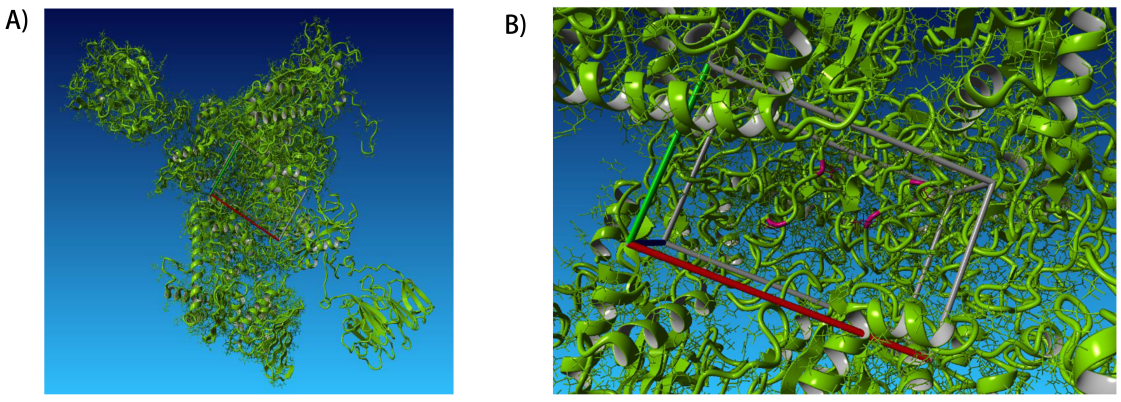

Figure 1. The predicted 3D structure of *K. pneumoniae gyrase* complex. A). The *K. pneumoniae gyrase* complex's predicted structure is shown. B) Mutated residues in the target site of S83L_D87H mutant *K. pneumoniae gyrase* are marked in red.

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 *E. coli* (n = 28) and *K. pneumoniae* (n = 44). The other identified bacteria were 11 *Proteus* spp., 10 *Enterobacter* spp., 4 *Pseudomonas* spp., and 3 *Staphylococcus* spp.

Quinolone/fluoroquinolone susceptibility profiling

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

Phenotypic and genotypic assessment of PMQR genes

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

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

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.

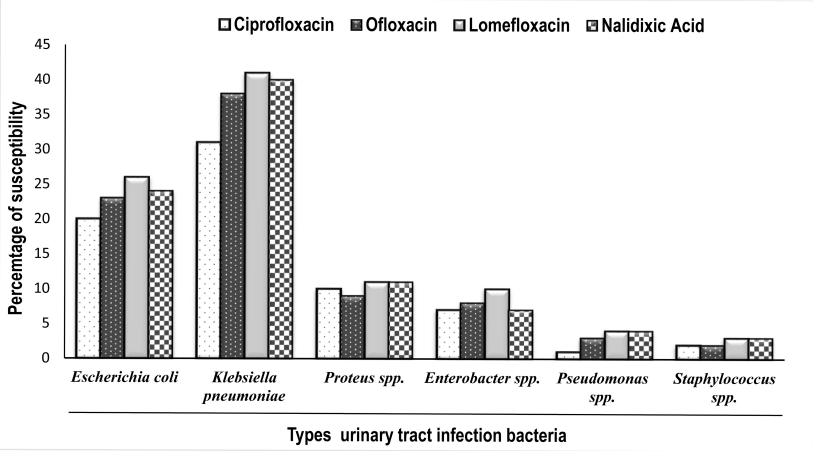

Figure 2. The binding energy of quinolones as ligands for docking into gyrase protein within the QRDRs.

any statistically significant correlation with PMQR genes carried by the UTI pathogens with the phenotypic resistance phenomena of any 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).

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 *K. pneumoniae* strain. We examined *gyrA* gene sequences of four isolates carrying the PMQR (*qnrS*) gene. Two isolates were *Proteus* spp., *E. coli*, and *K. pneumoniae*. No mutation was observed in the *gyrA* region in any of the four isolates (Figure 3A). Of the other three isolates, one *E. coli* and one *K. 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 *K. pneumoniae* showed a single mutation at S83L (Fig. 3B).

The amino acid alignment of the *gyrA* gene covering the QRDR of UTI pathogens, *K. pneumoniae* and *E. coli*, was related to those of reference *E. coli* ATCC 25922 strain and R4Y7H5 *K. pneumoniae* strain. Genetic divergence of the QRDR 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 (Fig. 3A). The amino acid sequences of the *gyrA* gene from the three bacterial isolates without PMQR genes were aligned (Fig. 3B). One UTI *E. coli* (QN4) and one *K. pneumoniae* (QN5) manifested double mutations at S83L (substitution of

Table 2. Identified PMQR genes, *qnrA*, *qnrB*, and *qnrS* in different UTI pathogens.

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

Table 3. Association of phenotypic quinolone susceptibilities with PMQR genes.

Quinolone susceptibility	Presence of PMQR genes[a] (*n* = 100)		*p* value [*]
	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 genes: plasmid-mediated quinolone-resistant genes, either or all of *qnrA*, *qnrB*, and *qnrS*.
[*] *p* value was calculated using the Cchi-squared statistic.

serine to leucine at position 83) and D87N (substitution of aspartic acid to asparagine at position 87). Another *K. pneumoniae* (QN7) showed a single mutation at S83L.

The 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 shown in Fig. 4. The required binding energy was 8.055 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, respectively (Fig. 4A).

Similarly, docking results were calculated in the case of *K. pneumoniae* as well. The binding affinity of ciprofloxacin for wild-type *gyrA* of *K. 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 (Fig. 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 and 6.957 kcal/mol for mutated strains with a single mutation at S83L and double mutations at S83L and D87N, respectively (Fig. 4B**)**. Docking research revealed that the displacement of the 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.

The comparative binding energy for ciprofloxacin and ofloxacin to wild-type gyrase and mutant gyrase are shown in Figure 4. Figure 4A shows the binding energy of ciprofloxacin and ofloxacin for the wild-type gyrase protein complex and double-mutation (S83L and D87N) gyrase proteins of *E. coli*. Figure 4B shows the adhesive strengths of ciprofloxacin and ofloxacin for both single- (S83L) and double-mutation (S83L and D87N) gyrase proteins *K. pneumoniae* when compared with that wild type. For all cases, binding affinity was detected lower in the case of mutated gyrase proteins.

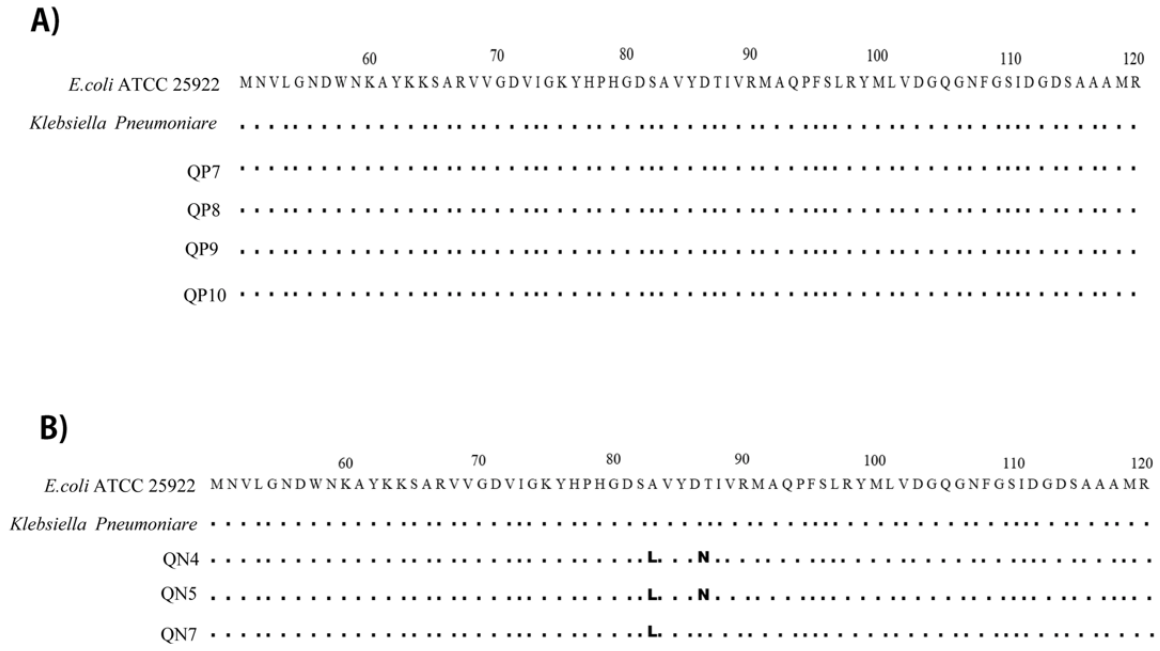

Figure 3. TAnalyses of mutations in the *gyrA* gene within the QRDRs.

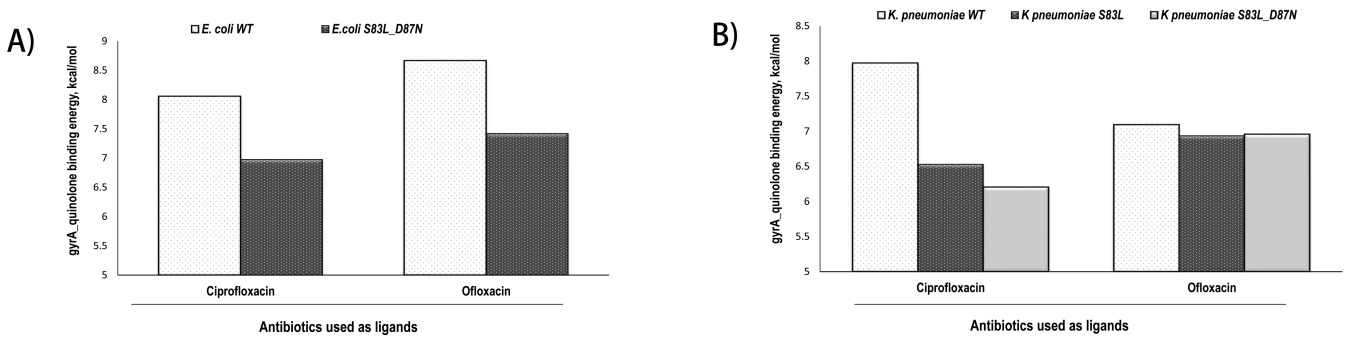

Figure 4. The binding energy of quinolones as ligands for docking into gyrase protein within the QRDRs.

DISCUSSION

This study investigated phenotypic quinolone/fluoroquinolone susceptibility and carriage of three PMQR genes, namely *qnrA, qnrB,* and *qnrS,* in UTI bacteria in Bangladesh. Furthermore, 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, which is similar to many other studies conducted in different countries (Cao *et al*., 2011; Colodner *et al*., 2008; Kim *et al*., 2020; Lee *et al*., 2018; Santiso *et al*., 2009). Ciprofloxacin was found to be the drug of 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 (Britto *et al*., 2018; Hooda *et al*., 2019; Saksena *et al*., 2018; Sedighi *et al*., 2015; Singh *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 was 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 relationship 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).

Furthermore, this study characterized the *gyrA* gene mutation mediated quinolone-resistant mechanisms in circulating UTI pathogens in Bangladesh. We found one *E. coli* and *K. pneumoniae* with two substitutions (S83L and D87N) and one *K. pneumoniae* with one mutation (S83L) in the *gyrA* gene. Similar mutations were reported in some diarrheal enterotoxigenic *E. coli* in Bangladesh (Begum *et al*., 2016) as well as UTI pathogens from other countries (Betitra *et al*., 2014; Komp 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 *K. pneumoniae* provided the principle of mutation-based QRDR (Ruiz, 2003). Mutated *gyrA* protein showed reduced ligand binding energy for both uropathogens, as observed in previous research reports (Chu *et al*., 2020; Varughese *et al*., 2018).

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 *K. pneumoniae* and *E. coli* were the most typical pathogens causing complicated and uncomplicated UTIs, which is similar to other studies (Founou *et al*., 2017; Hofer, 2019; Haque and Godman, 2021b; Urmi *et al*., 2020). 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 empirical 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 (Ara *et al*., 2021; Moran *et al*., 2020; Smith *et al*., 2018; Urmi *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, PMQR 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.

CONCLUSION

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 help 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 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.

CONFLICT OF INTEREST

None.

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CONSENT TO PARTICIPATE

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

AUTHOR CONTRIBUTIONS

Conceptualization: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Data curation: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Formal analysis: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Funding acquisition: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Methodology: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Project administration: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Supervision: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Visualization: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Writing–original draft: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG; Writing–review and editing: TAN, ULU, ABMMKI, BA, SN, ASMM, HL, SK, DJ, NAAR, MH, SI, BG.

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 supporting the findings of this study will be made available by the corresponding author, SI, upon reasonable request

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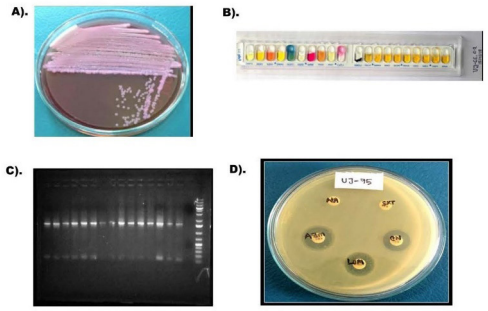
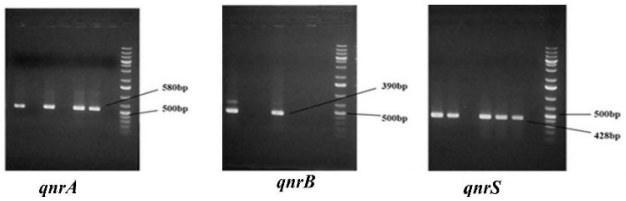
Supplementary Table 1. Dispersal of PMQR genes and phenotypic quinolone susceptibilities of UTI pathogens.

Quinolone resistance trait	Pathogens (n = 100)	Phenotypic susceptibility frequency of isolates to four quinolone antibiotics											
		Nalidixic acid			Lomefloxacin			Ofloxacin			Ciprofloxacin		
		S	R	*p* value *	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	

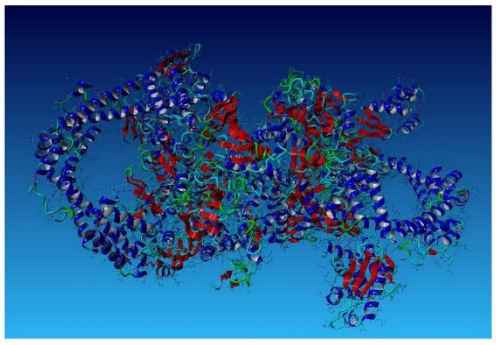
S = sensitive; R = resistant.
[a] PMQR genes: plasmid-mediated quinolone-resistant genes, *qnrA*, *qnrB*, and *qnrS*.
* *p* value was calculated using the Cchi-squared statistic.



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 manufactures' instructions. C) Amplification of 16s rDNA gene for confirmed identification. D) Phenotypic susceptibilty analysis of quinolone antibitics to the UTI pathogenes.



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



Supplementary Figure 2. *Escherichia coli* gyrase complex bound to inhibitor YacG (PDB ID:4TMA).