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Detection of Quinolone Resistance *qnr* genes and its Association with Carbapenemase genes in *qnr* Positive Enterobacteriaceae in a Tertiary Hospital in Bangladesh

Bithi Das[1], Tapan Kumar Das[2], Hosne Jahan[3], Nazmun Sharmin[4], Nasrin Zahan[5], Ishrat Sharmin[6]

[1]Assistant Professor, Department of Microbiology, National Institute of Cardiovascular Diseases, Dhaka, Bangladesh; [2]Assistant Registrar, Department of Cardiology, National Institute of Cardiovascular Diseases, Dhaka, Bangladesh; [3]Associate Professor, Department of Microbiology, National Institute of Cardiovascular Diseases, Dhaka, Bangladesh; [4]Curator, Department of Microbiology, National Institute of Cardiovascular Diseases. Dhaka, Bangladesh; [5]Microbiologist, Department of Microbiology, National Institute of Cardiovascular Diseases, Dhaka, Bangladesh; [6]Associate Professor, Department of Microbiology, National Institute of Cardiovascular Diseases, Dhaka, Bangladesh

Abstract

Background: Multidrug resistance in Enterobacteriaceae is dramatically increasing worldwide day by day. **Objective:** The purpose of the present study was to detect the prevalence of quinolone resistance *qnr* genes and its association with carbapenemase genes in qnr positive Enterobacteriaceae. **Methodology:** This cross-sectional study was conducted from January 2015 to December 2015 at Dhaka Medical College Hospital, Dhaka, Bangladesh. A total of 270 Enterobacteriaceae were collected from inpatient and outpatient departments of DMCH irrespective of age, sex and antibiotic intake analyzed for antibiotic susceptibility. The *qnr* determinant screening among ciprofloxacin resistant strains were conducted using PCR amplification. Searching of carbapenemase genes in *qnr* positive Enterobacteriaceae were conducted also by using PCR. **Results:** The *qnr* gene was detected in 141 (62.67%) of the 225 quinolone resistant isolates by using PCR. Highest proportion of *qnrS* were detected followed by *qnrB* and *qnrA* from quinolone resistant strains. *qnrB* genes were co-existed with *NDM-1, VIM, IMP* and *KPC* genes. *qnrS* genes were also associated with *NDM-1, VIM, IMP* and *KPC* genes. Antimicrobial-resistance rates of Enterobacteriaceae to ciprofloxacin was 83.33%. **Conclusion:** The *qnr* genes were highly prevalent in Enterobacteriaceae. They were closely associated with carbapenemase genes.

Keywords: Bangladesh; Carbapenemase genes; *qnr* genes

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Introduction

Quinolones are synthetic antimicrobial agents that are extensively used in medical applications throughout the world[1]. The most commonly used fluoroquinolone named ciprofloxacin, which was introduced in 1987[2]. Ciprofloxacin is mainly used against gram-negative bacteria such as Enterobacteriaceae, *Pseudomonas aeruginosa, Haemophilus influenzae* and *Moraxella catarrhalis*; however, it displays less activity against gram-positive infections[3]. However, owing to the widespread use of fluoroquinolone in human, the resistance rate of these antimicrobial agents has risen in all bacterial species. In the case of UTI patients, the rate of fluoroquinolone resistance was reported in the range of 6.3% to 62.0% in gram-negative strains[4].

Resistance to quinolone is generally due to chromosomal mutation[5]. Recently, a multi-resistance plasmid is discovered that encodes transferable resistance to quinolones; its prevalence has been increasingly described among Enterobacteriaceae in recent decades[6]. Plasmids have a crucial role in the dissemination of drug resistance genes like plasmid mediated quinolone resistant *(PMQR)* genes, extended spectrum β-lactamase (ESBL) genes, AmpC β-lactamase genes and carbapenemase genes[7]. Plasmid-mediated quinolone resistance *(PMQR)*

Correspondence: Dr Bithi Das, Assistant Professor, Department of Microbiology, National Institute of Cardiovascular Diseases, Sher-E-Bangla Nagar, Dhaka 1207, Bangladesh; Email: bithidasm40@gmail.com; Cell No.: +8801712174071;
ORCID: https://orcid.org/0009-0008-5071-8949

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determinant, *qnr* genes have been mostly identified in clinical isolates of Enterobacteriaceae and have been shown to play not only an important role in quinolone resistance but also this drug resistance gene can disseminate from one bacterium to another. Thus, presence of *PMQR* genes among Enterobacteriaceae provides a wider reservoir for the spread of these organisms[8]. All types of qnr determinants have been reported in various Enterobacteriaceae and were frequently found in *Escherichia coli*, followed by *Klebsiella species, Enterobacter species* and S*almonella spp.* from clinical isolates around the world[9]. In addition, they have been detected less frequently in non-fermenters such as P*seudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii*[10].

Simultaneous presence of qnr *genes* and other drug resistance genes such as extended-spectrum β-lactamase, AmpC β-lactamase and carbapenemase on the same plasmid causing multidrug failure[7]. *qnrB* has been co-existed with *KPC* and *IMP* genes[11]. *qnrS* is associated with *IMP* gene[12]. Few studies have been carried out in detecting *qnr* genes among *Shigella flexneri* from clinical samples and *Esch. coli* from water samples in Bangladesh[13,14]. Therefore, this study was designed to explore quinolone resistant Enterobacteriaceae from different specimens followed by searching the prevalence of three groups of quinolone resistance genes (*qnrA, qnrB & qnrS*) by PCR. Then genes for carbapenemase were detected in qnr positive strains to observe the association of these genes with *qnr* genes in developing multiple drug resistance.

Methodology

Study Settings and Population: This cross-sectional study was conducted from January 2015 to December 2015 at Dhaka Medical College Hospital, Dhaka, Bangladesh. A total of 270 Enterobacteriaceae were isolated from 340 samples collecting from inpatient and outpatient departments of DMCH irrespective of age, sex and antibiotic intake. This study was approved by the Ethical Review Committee of Dhaka Medical College and informed written consent was taken from each participant.

Study Procedure: Sampling locations included urine (N=96), wound swab (N=62), sputum (N=58) and blood (N=54). All the wound swab, sputum and urine samples were inoculated in blood agar and MacConkey agar media and incubated at 37°C aerobically for 24 hours. Incubated plates were then examined for the presence of colonies of bacteria. Primary blood culture was done in Trypticase soyabroth then subculture on blood agar and MacConkey agar media. Smear was prepared from sample and culture plate and stained by Gram's stain as per standard procedure and were examined under microscope for the presence of gram positive or gram-negative organisms. All the isolated organisms were identified by their colonial morphology, gram stain characteristic and relevant biochemical tests[15]. *Escherichia coli* grew as smooth, glossy, pink colonies on MacConkey agar media, gram negative rod, motile and characteristic sugar fermentation tests, indole positive, citrate non-utilizer. *Klebsiella species* were lactose fermenting mucoid pink colony in MacConkey agar media, acidic (yellow) slant and butt with gas production but no H_2S in TSI agar, urease positive, non-motile. Citrobacter species are late lactose fermenter, motile with citrate positive. Proteus species were pale colony on MacConkey agar media, characteristic swarming growth on blood agar media and 'fishy' odour, rapid urea hydrolysis in MIU media, motile, slant (red) butt (yellow) with H_2S production. *Enterobacter species* were pink colony in MacConkey agar media, motile, oxidase negative, citrate utilizer, indole negative, urease negative, gas producing, no hydrogen sulphide production in TSI agar media.

Antimicrobial susceptibility test[16]: Susceptibility to antimicrobial agents of all isolates was done by Kirby Bauer modified disk diffusion technique using Muller Hinton agar plates and zones of inhibition were interpreted according to CLSI guidelines (CLSI, 2014). Antibiotic disks such as ciprofloxacin (5 µg), cefepime (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), amoxiclav (amoxicillin 20 µg & clavulanic acid 10 µg), amikacin (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), cefoxitin (30 µg) and cotrimoxazole (1.25/23.75 µg). Screening of quinolone resistant Enterobacteriaceae was done during disk-diffusion method using ciprofloxacin disk. The minimum inhibitory concentration (MIC) of ciprofloxacin and imipenem were determined by agar dilution method. Escherichia coli ATCC 25922 was used as control strain to assess the performance of the method. Drug resistant organisms were classified as multidrug-resistant, extensively drug-resistant and pandrug-resistant. Multidrug- resistant was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (such as, aminoglycosides, tetracycline, carbapenem,

cephalosporine, fluoroquinolones, phosphonic acids, glycylcycline, monobactams, polymyxins). Extensively drug-resistant was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories). Pandrug-resistant was defined as non-susceptibility to all agents in all antimicrobial categories[17].

Phenotypic detection of carbapenemase producers: All the isolates showing reduced susceptibility to imipenem (zone diameter< 19mm) were tested for carbapenemase production using the Modified Hodge Test. Briefly, a lawn culture of 1:10 dilution of 0.5 McFarland's standard *Esch. coli* ATCC 25922 broth was done on a Mueller-Hinton agar plate. A 10-µg imipenem disk was placed in the centre of the plate. Then, imipenem resistant test strain was streaked from the edge of the disk to the periphery of the plate in three different directions. After overnight incubation, the plates were observed for the presence of a clover leaf shaped zone of inhibition and the plates with such zones were interpreted as Modified Hodge test positive[18]. Double–disk synergy test (DDS) test and combined disk (CD) assays were also performed to screen MBLs producers. During DDS test, sterile cotton swab & test inoculums (compared with McFarland standard) were inoculated in Mueller-Hinton agar plates. Imipenem disc was placed on the inoculated plate. A blank disc containing 20 µl of Tris-EDTA (1.0 M Tris-HCL, 0.1 M EDTA, pH8.0) and 20 µl of 1:320 diluted 2-mercaptopropionic acid were placed 10 mm apart from the imipenem disk. Inoculated Mueller-Hinton agar plate was incubated at 37ºC for 24 hours. A clear extension of the edge of the inhibition zone of imipenem disc towards Tris-EDTA-MPA disc was interpreted as MBLs production[19]. During CD assay, Mueller-Hinton agar plate was inoculated with the 0.5 McFarland bacterial suspensions and two imipenem discs were placed on it. One imipenem disc was supplemented with 5 µl of 0.5 M EDTA solution as the disc contains approximately 750 µg EDTA. Inoculating plate was incubated at 37ºC for 24 hours. An increased zone diameter of ≥6 mm around the disc containing imipenem supplemented with 750 µg EDTA compared with disc containing imipenem alone was suggested as MBLs production[20].

PCR for detection of qnr and other genes in *qnr* positive quinolone resistant strains: Genomic DNA of the ciprofloxacin resistant bacterial strains was amplified by PCR for the detection of three groups of Qnr determinants (qnrA, qnrB and qnrS). Briefly, colonies were suspended in 50 µL of water in a microcentrifuge tube and boiled to prepare DNA templates.PCR reaction consisted of preheat at 94ºC for 10 minutes followed by 36 cycles of denaturation at 94ºC for 45 seconds, annealing at 530 C for 45 seconds, extension at 72ºC for one minute with a final extension at 720 C for 10 minutes. Reaction mixtures without a DNA template served as negative controls. PCR was used for identification of carbapenemase encoding genes (NDM-1, KPC, IMP, VIM) among qnr positive strains (Table 1).

Table 1: Primers for genes of qnr and Carbapenemase Used In This Study

Genes	Sequence (5′-3′)	Bp
QnrA-F	ATTTCTCACGCCAGGATTTG	516
QnrA-R	GATCGGCAAAGGTTAGGTCA	
QnrB-F	GATCGTGAAAGCCAGAAAGG	469
QnrB-R	ACGATGCCTGGTAGTTGTCC	
QnrS-F	ACGACATTCGTCAACTGCAA	417
QnrS-R	TAAATTGGCACCCTGTAGGC	
NDM1-F	ACCGCCTGGACCGATGACCA	264
NDM1-R	GCCAAAGTTGGGCGCGGTTG	
IMP-F	GGAATA GAGTGGCTTAAYTCTC	188
IMP-R	CCAAACYACTASGTTATCT	
VIM-F	GATGGTGTTTGGTCGCATA	390
VIM-R	CGAATGCGCAGCACCAG	
KPC-F	CGTCTAGTTCTGCTGTCTTG	498
KPC-R	CTTGTCATCCTTGTTAGGCG	

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Continuous data were expressed as mean, standard deviation, minimum and maximum. Categorical data were summarized in terms of frequency counts and percentages.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Formal ethics approval was granted by the local ethics committee. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

Out of 270 (71.05%) Enterobacteriaceae, Esch. coli was the predominant strains (n =127, 47.04%) (Table 2).

Total 225 (83.33%) Enterobacteriaceae showed ciprofloxacin resistance. The qnr gene was detected in 141 (62.67%) of the 225 quinolone resistant isolates by using PCR. Total 187 qnr genes were isolated. More than one genes were present in many organisms. Highest proportion of qnrS (n =84,59.57%) were detected followed by qnrB (n =70, 49.64%) and qnrA (n =33, 23.40%) from quinolone resistant strains. qnrA and qnrS were highly prevalent in Esch. coli. On the other hand, qnrB was highly prevalent in Klebsiella spp. (Table 3).

More than one type of qnr genes were present in most of the organisms (Figure I).

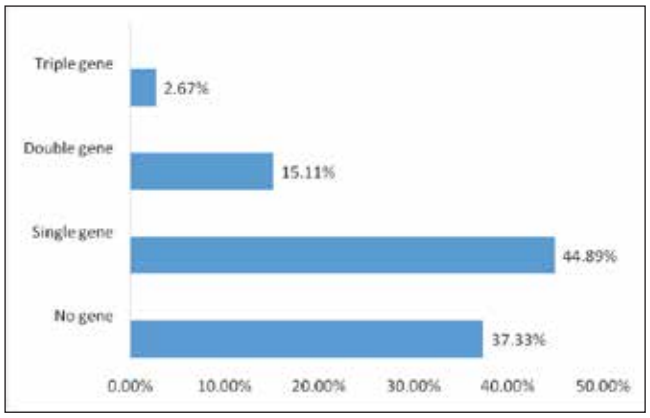

Figure I: Distribution of qnr encoding genes in quinolone resistant Enterobacteriaceae (N=225)

In the present study, 22 carbapenemase producers were identified among 141 qnr positive strains. Eighteencarbapenemase producers were positive for carbapenemase encoding genes detected by PCR. Total

Table 2: Distribution of Enterobacteriaceae isolated from different samples (N=270)

Enterobacteriaceae	Urine	Blood	Sputum	Wound swab	Total
Esch. coli	62(64.6%)	25 (46.30%)	21(36.21%)	19(30.64%)	127 (47.04%)
Klebsiella species	12(12.50%)	24 (44.44%)	31(53.45%)	20(32.26%)	87 (32.22%)
Citrobacter species	8 (8.34%)	3 (5.56%)	3(5.17%)	4(6.45%)	18 (6.67%)
Proteus species	6(6.25%)	1 (1.85%)	1(1.72%)	9 (14.52%)	17 (6.29%)
Enterobacter species	8(8.33%)	1(1.85%)	2(3.45%)	10 (16.13%)	21 (7.78%)
Total	**96 (100%)**	**54 (100%)**	**58 (100%)**	**62 (100.0%)**	**270 (100%)**

Table 3: Prevalence of different *qnr* genes among *qnr* positive Enterobacteriaceae (N=141)

Enterobacteriaceae	*qnrA*	*qnrB*	*qnrS*	*qnr* +ve strains
Esch. coli (N=71)	17+2* (26.76%)	20+2* (30.98%)	44+2* (64.79%)	50.4%
Klebsiella species N=50)	6+4* (20.00%)	38+4* (84.0%)	24+4* (56.0%)	35.5%
Citrobacter species (N=10)	1(10.0%)	2(20.0%)	7(70.0%)	7.0%
Proteus species (N=2)	0(00.0%)	1(50.0%)	1(50.0%)	1.4%
Enterobacter species (N=8)	3(37.5%)	3(37.5%)	2(25.0%)	5.7%
Total	**33 (23.4%)**	**70(49.6%)**	**84 (59.6%)**	**100.0%**

Note: N= Total number of qnr positive Enterobacteriaceae, n = Number of qnr genes; '*' denotes positive for all varieties of qnr genes

Table 4: Prevalence of carbapenemase encoding genes among different species of imipenem resistant *qnr* positive Enterobacteriaceae (N=22)

Imipenem resistant	Carbapenemase encoding genes			
Enterobacteriaceae	*NDM-1*	*VIM*	*IMP*	*KPC*
Esch. coli (N=13)	9 (69.23%)	2 (15.38%)	3 (23.08%)	3 (23.08%)
Klebsiella species (N=7)	6 (85.71%)	2 (28.57%)	0 (0.00%)	2 (28.57%)
Citrobacter species (N=1)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Enterobacter species (N=1)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Total	**15 (68.18%)**	**4 (18.18%)**	**3 (13.64%)**	**5 (22.73%)**

Note: N= Total number of imipenem resistant Qnr positive Enterobacteriaceae, n = Number of carbapenemase encoding genes

27 carbapenemaseencoding genes were isolated. Highest proportion of *NDM-1* (n= 15, 68.18%) were detected followed by *KPC* (n=5,22.73%), *VIM* (n=4,18.18%), *IMP* (n=3,13.64%). All the genes were highly prevalent in *Esch. coli* (Table 4).

Table 5: Distribution of carbapenemase encoding genes among different qnr genes of carbapenemase producers (N=20)

qnr Genes	*NDM-1*	*VIM*	*IMP*	*KPC*
qnr A (N=3)	0 (0.00%)	0(0.00%)	0(0.00%)	0(0.00%)
qnrB (N=5)	4 (80.00%)	2(40.00%)	2(40.00%)	3(60.00%)
qnrS (N=12)	11(91.67%)	2(16.67%)	1(8.33%)	2(16.67%)
Total	**15(75.00%)**	**4(20.00%)**	**3(15.00%)**	**5(25.00%)**

Note: N = Total number of qnr genes in carbapenemase producers, n = Number of carbapenemase encoding genes among *Qnr* genes

qnrB and qnrS had been coexisted with genes for carbapenemase such as *NDM-1, KPC, IMP* and *VIM* genes. No carbapenemase encoding genes were found with *qnrA* (Table 5).

Table 6. Comparison of results of DDS test, CD assay & MHT with PCR for detection of MBL producers among The Imipenem Resistant *qnr* Positive Enterobacteriaceae (N=22)

DDS	PCR		Total
	Positive	Negative	
Positive	11 (50.00%)	2 (9.09%)	13 (59.09%)
Negative	7 (31.82%)	2 (9.09%)	9 (40.91%)
CD assay			
Positive	12 (54.54%)	3 (13.64%)	15 (68.18%)
Negative	6 (27.28%)	1 (4.54%)	7 (31.82%)
MHT			
Positive	4 (18.18%)	3 (13.64%)	7 (31.82%)
Negative	14 (63.64%)	1 (4.54%)	15 (68.18%)

The DDS test, CD assay and MHT detected 13(59.09%), 15(68.18%) and 7(31.82%) carbapenemase producers respectively among the 22 imipenem-resistant isolates. Out of the four negative amplified PCR products, two were positive by the DDS test and three were positive by CD assay and MHT. Considering the PCR as the gold standard, the sensitivity of the DDS test, CD assay and MHT were 61.11%, 66.67% and 22.22% respectively; specificity was 50%, 75.02% and 24.97% respectively (Table 6).

Discussion

There is an increasing trend of quinolone resistant organisms all over the word. In consistent with other report of DMCH[21] quinolone resistant Enterobacteriaceae were 83.33% in this study. In contrast to the present findings, 27.7% quinolone resistant organisms were reported from Korea[11].

Factors such as widespread indiscriminate use of drugs, truncated antimicrobial therapy, inadequate access to effective drugs and sometimes drugs of questionable quality are likely to be contributing factors for high resistance rate to ciprofloxacin in the present study[22-23]. For this reason, high proportion (62.67%) of *qnr* positive Enterobacteriaceae were observed in this study. High MIC values (8 to ≥128 μ g/ml) were also found for ciprofloxacin in this study which might be due to the same reason[24].

qnr gene was absent in 37.33% of the quinolone resistant isolates. This might be due to possibility of presence of other variety of qnr genes such as, *qnrC* and *qnrD* genes or presence of another genes such as AAC (6′)-Ib gene that causing drug modification[25] or genes for quinolone efflux pumps (*qepA and oqxAB*) that enhanced efflux of drugs[26]. *qnrA* and *qnrS* were highly prevalent in *Esch. coli.* and *qnrB* was highly prevalent in *Klebsiella spp.* in the present study and these results were consistent with other report[11].

Total 22 carbapenemase producers were identified among 141 *qnr* positive strains. Carbapenemase producing organisms isolated from imipenem resistant *qnr* positive Enterobacteriaceae were 84.2% cases[21] followed by 81.8% in the present study. High prevalence of carbapenemase producing strains are found among Enterobacteriaceae because of the presence of carbapenemase-encoding genes on plasmid along with other resistance genes such as *qnr* resulting in multidrug-resistant, extremely drug-resistant and pandrug-resistant bacteria[27].

In the present study, 68.2% isolates were positive for *NDM-1* among 22 imipenem resistant strains. The prevalence of *NDM-1* producers is increasing in Bangladesh which is reflected by another study conducted in DMCH which was 55%[21]. In Indian studies, the prevalence of *NDM-1* producers among carbapenem resistant Enterobacteriaceae ranged between 31.2% and 91.6%28. *NDM-1* producing organisms are now alarmingly rising worldwide and pose therapeutic failure[29]. The high prevalence of *NDM-1* type carbapenemase (85.78%) among Klebsiella was identified in the present study. In a study in DMCH, it was reported that 80% Klebsiella were positive for *NDM-1*[21]. Another study in India it was reported that *K. pneumoniae* and *Esch. coli* were the most frequent bacteria for *NDM-1* production[30]. Rapid dissemination of *NDM-1* producing organisms might be facilitated by the conditions like overcrowding, over-the-counter availability of antibiotics, low level of hygiene, and weak hospital

antibiotic policies[31].

In this study, the high prevalence of KPC type carbapenemase (22.73%) were identified among 22 imipenem resistant strains. In a study in DMCH, it was reported that 20% *Klebsiella spp.* were positive for *KPC*[20]. This suggests the high prevalence of *KPC* genes among Enterobacteriaceae in Bangladesh. The presence of this gene suggests the possibility of horizontal transmission, as this carbapenemase has been associated with mobile genetic elements (transposons) which can be transferred from one bacterium to another[32].

In consistent with other report from DMCH[21], 4 (18.18%) isolates were positive for *VIM* and 3 (13.64%) isolates were positive for *IMP* among 22 imipenem resistant strains in this study. In contrast to the present findings, 100% *VIM-1* producers were identified from Greece[33]. The proportion of *MBL* producers from different studies including the present one suggests that the prevalence of *MBL* producers varies with geographical areas and time.

In this study, 4 (18.19%) carbapenemase resistant isolates had no carbapenemase encoding genes. This may be due to presence of other carbapenemase encoding genes such as *OXA-23, OXA-48, OXA-181* types that was not included in the current study[34,35]. Enterobacteriaceae have the capacity to elude the action of carbapenems through modification of outer membrane permeability such as porin loss, up-regulation of efflux systems, production of carbapenem hydrolyzing β-lactamases (i.e. hyperproduction of AmpC β-lactamases, certain *ESBLs* with increased capacity to hydrolyze carbapenems) and production of carbapenemases either serine based carbapenemases or *MBLs*[36].

In this study, *qnrB* and *qnrS* had been coexisted with genes for carbapenemase such as *NDM-1, VIM, IMP* & *KPC.NDM-1* were highly prevalent among qnrS and *qnrB* genes, no carbapenemase encoding genes were found with *qnrA*. In a study in Hong Kong, it was found that *qnrB* had been also co-existed with *KPC* and *IMP*[37].

In this study, MIC of imipenem among imipenem resistant *qnr* positive Enterobacteriaceae ranged from 4 to ≥128 µg/ml. The variation in the MIC values for carbapenems may be influenced by type and expression of carbapenemase enzymes, the bacterial species, the presence of other resistance mechanism (such as, Extended spectrum and AmpC β-lactamase), reduced permeability and or efflux pump[38] which is similar to the present study.

Conclusion

The presence of significant proportion of carbapenemase producing Enterobacteriaceae among *qnr* positive strains highlights the emerging therapeutic challenge in Bangladesh. So detection of multiple genes in an organism will be helpful in selection of appropriate antimicrobial strategics which will reduce the emergence and spread of *MDR* Enterobacteriaceae.

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

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Authors' contributions
Das B, Jahan H conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Jahan H, Sharmin N, Das TK contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Sharmin N, Das TK, Zahan N, Sharmin I involved in the manuscript review and editing. Das B as collector of Data and Data Analyst. All authors read and approved the final manuscript.

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

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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ORCID
Bithi Das: https://orcid.org/0009-0008-5071-8949
Tapan Kumar Das: https://orcid.org/0009-0000-0851-4274
Hosne Jahan: https://orcid.org/0009-0000-9356-0768
Nazmun Sharmin: https://orcid.org/0000-0003-4258-3868
Nasrin Zahan: https://orcid.org/0009-0000-4943-8554
Ishrat Sharmin: https://orcid.org/0009-0004-1996-5758

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