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ORIGINAL PAPER

Ciprofloxacin-resistant *Escherichia coli* in hospital wastewater of Bangladesh and prediction of its mechanism of resistance

Farhima Akter · M. Ruhul Amin · Khan Tanjid Osman · M. Nural Anwar · M. Manjurul Karim · M. Anwar Hossain

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Abstract Hospital and agriculture wastewater is mostly responsible for causing environmental pollution by spreading un-metabolized antibiotics and resistant bacteria, especially in Bangladesh. Here, we studied the influence of the most frequently prescribed antibiotic, fluoroquinolone (~72%), on the development of antibiotic resistance in *Escherichia coli*. Out of 300, 24 ciprofloxacin resistant *E. coli* isolates were selected for the study that showed the MBC_{100} higher than expected (600 μg/mL). Here, we profiled plasmid, sequenced *gyr* genes, screened mutations and analyzed the effect of mutation on drug-protein interaction through molecular docking approach. We found that (1) out of 10, most of them (n = 7) had large plasmid(s); (2) all ciprofloxacin-resistant isolates had *gyrA* double mutations (S83L and D87Y); (3) no isolate had *qnr* gene; and (4) docking of ciprofloxacin with DNA gyrase A subunit suggests that acquisition of double mutation leads to alteration of the ciprofloxacin binding pocket.

Keywords Hospital wastewater · Antibiotic resistant bacteria · Ciprofloxacin · DNA gyrase A

Farhima Akter, M. Ruhul Amin and Khan Tanjid Osman contributed equally.

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F. Akter · M. R. Amin · M. M. Karim · M. A. Hossain (✉)
Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh
e-mail: anwar5533@yahoo.com

K. T. Osman · M. N. Anwar
Department of Microbiology, University of Chittagong, Chittagong 4331, Bangladesh

Introduction

There has been growing concern about antimicrobial resistance in the environment and formation of biological resistant biofilm and their ecological effects (Kummerer 2001, 2003), especially in rural Bangladesh and other developing countries (Hossain et al. 1998; Sack et al. 1997; Hoge et al. 1998). The hospital wastewater carries both the resistant bacteria (Larson 2007) and un-metabolized antibiotic. In Bangladesh, the wastewater pollution is more alarming due to direct disposal of hospital and agricultural wastewater to the environment (Al-Ahmad et al. 1999).

Currently, the management of bacterial infections has been increasingly complicated due to the emergence of resistance to most first-line antimicrobial agents, including fluoroquinolones, penicillins and cephalosporins (Karlowsky et al. 2001; Urban et al. 2003; Obritsch et al. 2004; Paterson et al. 2004). It has also become evident that this antibiotic is extensively used and misused in hospitals, communities, as well as in veterinary medicine (Blondeau and Yaschuk 1996; Acar and Goldstein 1997). However, it has been uncovered recently that there are clear correlations between fluoroquinolone resistance in *Escherichia coli* isolates and hospital location, economic status, and culture site (Boyd 2008).

Targets of quinolones are DNA gyrase and topoisomerase IV that are tetrameric A_2B_2 enzymes encoded by the *gyrA* and *gyrB,* and the *parC* and *parE* genes, respectively. Resistance to fluoroquinolone usually results from chromosomal mutations (Wang et al. 2004). In Gram negative organisms, mutations in chromosomal *gyrA* were reported to produce low level of resistance, whereas higher MICs are associated with additional mutations, particularly in *parC,* but also in *gyrB* and, less commonly, in *parE* (Woodford and Ellington 2007). *gyrA* mutations in the

Ser-83 and Asp-87 codons were most frequently associated with fluoroquinolone resistance in *E. coli* (Weigel et al. 1998). The Ser83Leu mutation was consistent for all fluoroquinolone-resistant isolates, and in isolates with double mutations, the second site, Asp-87 was substituted for Gly, Tyr, or Asn. Plasmid-mediated quinolone resistance has also been reported and a resistance gene, *qnr*, was found on plasmids varying in sizes ranging from 54 to 180 kb in clinical isolates of *Klebsiella pneumoniae* and *E. coli* (Wang et al. 2004).

In Bangladesh, no study had been conducted concerning the effects of hospital effluents on the emergence and development of drug-resistant bacteria. We, for the first time, investigated the level of antibiotics and other drugs used in medicine and agriculture/aquaculture and their effects on natural microbial communities. Hence, the aims of the present study were (1) to address the presence of resistant bacteria in hospital waste discharge, (2) to correlate between the antibiotics used by the patients and resistance developed in bacteria, and (3) to predict the mechanism that confers ciprofloxacin resistance in the isolated *E. coli.*

Materials and methods

Collection of samples

Five groups of samples were collected from the sites as depicted in Fig. 1. Total three groups of samples connected with Chittagong Medical College Hospital (CMCH) liquid waste and two control groups of samples, indirectly-connected (control 1) and not connected (control 2) with CMCH wastewater were collected within a two month period, August–September 2006. Sampling (300 mL) was done in triplicates and were collected three times over the stated period. Samples were grouped on the basis of the collection sites and maintained at 4°C during transport.

Bacteriological enumeration

For total bacterial count, serial tenfold dilutions (up to 10^{-6}) of samples were prepared in physiological saline, and 1 mL aliquots were poured on Plate Count Agar (PCA). Plates were incubated for 24 h at 37°C before bacteriological counts were performed. For total resistant bacterial count, ciprofloxacin from the stock solution (10 mg/mL) was given in different concentrations in the molten PCA media at about 45–50°C.

Isolation of ciprofloxacin resistant *E. coli*

Single colonies from different samples were picked up by sterile tooth picks randomly from the ciprofloxacin-containing PCA plates. Initially 300 colonies were selected and were grown on Eosine Methylene Blue (EMB) agar plate containing 50 μg/mL ciprofloxacin and were then characterized using several biochemical tests according to the methods described by Cappucino (Cappucino and Sherman 1996). After the biochemical tests, twenty-four *E. coli* colonies were chosen for the present study.

Determination of multi-drug resistance pattern

To see the effect of antibiotics used in hospitals on the isolated *E. coli* strains, bacterial susceptibility to different

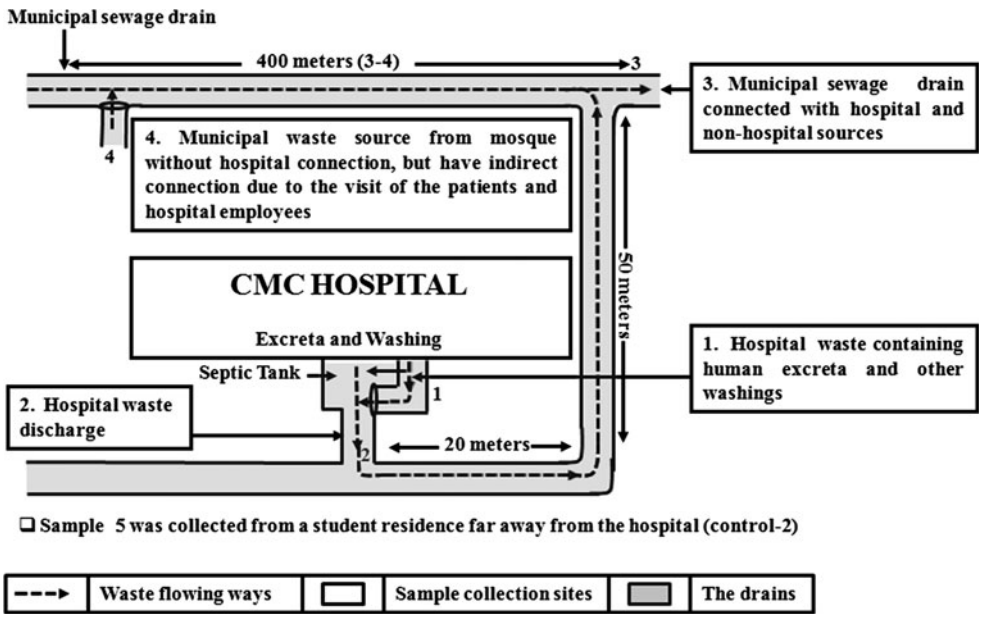

Fig. 1 Three groups of samples were collected from CMCH and the groups are indicated in numbers (*1*, *2*, and *3*). In addition, two control groups of samples, one indirectly connected with hospital wastewater (number *4* and control *1*), and the other from a student residence far away from the hospital and not connected with hospital wastewater (number *5* and control *2*) were collected. The wastes from different wards of the hospital meeting the municipal sewerage system are indicated

antimicrobial agents was measured in vitro by employing the modified Kirby-Bauer (Bauer et al. 1966) method by measuring zone sizes (in mm). Commercially available antibiotics discs (Oxoid, Basingstoke, UK) were used for the test. The antibiotic discs used in this study included ampicillin, bacitracin, cefalexin, ceftazidime, chloramphenicol, ciprofloxacin, cloxacillin, doxycycline, gentamycin, imipenem, kanamycin, nitrofurantoin, novobiocin, penicillin, spectinomycin, sulphamethoxazole and vancomycin. Susceptible *Escherichia coli* ATCC 25922 was used as a control.

Analysis of resistance pattern to ciprofloxacin

A single colony from overnight culture containing ciprofloxacin was inoculated in 3 mL Mueller–Hinton Broth containing ciprofloxacin (50 μg/mL) and was incubated overnight at 37°C at 150 rpm. Cultures were then transferred to 3 mL MHB (transfer rate was 1:200) containing different concentrations of ciprofloxacin (0, 100, 200, 300, 400, 500, and 600 μg/mL). More than 600 μg/mL could not be used because the ciprofloxacin precipitates out of the solution. After inoculation, culture preparations were incubated at 37°C for 4 h at 150 rpm. Optical density was measured at 600 nm. After serial dilution, total resistant bacterial count was determined by drop plate method. Percentage of decrease CFU was determined by using following formula: % decrease of CFU = [(CFU control—CFU antibiotic)/CFU control] × 100.

Plasmid isolation

Plasmid DNA was extracted according to the alkaline lysis method (Kado and Liu 1981) with modifications (Talukder et al. 2002). The molecular weight of the plasmid DNA was determined by comparison to the electrophoretic mobility of plasmids of known molecular weights using *E. coli* PDK-9 (Haider et al. 1989).

PCR amplification of *gyrA, gyrB* and *qnr*

Chromosomal DNA from *E. coli* isolates were extracted and purified by the method described earlier (Maniatis et al. 1989) with minor modifications (Ansaruzzaman et al. 2007). Primers used for the amplification of *gyrA, gyrB* and *qnr* genes are given in Table 1 [(Wang et al. 2004) for *gyrB*, (Martinez–Martinez et al. 2003) for *gyrA* and (Jeong et al. 2005) for *qnr*]. 25 μL of each reaction mixture contained 3.0 μL 10 × PCR buffer (Invitrogen), 0.6 μL dNTPs (Invitrogen), 12.5 pmol of oligonucleotide primer, (Integrated DNA Technologies, Inc. USA), 1 μL of chromosomal DNA and 0.3 μL of *Taq* DNA polymerase (Invitrogen). In case of *gyrA* and *gyrB*, the cycling program involved 30 cycles of 96°C for 30 s, 64°C for 30 s, and 72°C for 2 min. In case of *qnr*, the cycling program involved 30 cycles of 96°C for 30 s, 57°C for 30 s, and 72°C for 1 min. PCR products were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 mg/mL).

Nucleotide sequencing

The PCR amplicons were purified with the QIA quick Gel Extraction Kit and sequenced using an automated ABI prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence analysis and comparison with known sequences were performed with the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Nucleotide sequences accession number

The nucleotide sequences of *gyrA* were submitted to GenBank using the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) Sequin, version 5.26. Accession numbers for these strains were assigned as: GU166750 and GU166751.

Analysis of the impact of mutation in QRDR region of *E. coli* DNA gyrase

To understand the impact of mutation in S83L and D87Y, we have analyzed the crystal structure of the breakage-reunion domain of the DNA Gyrase A subunit obtained from the Protein Data Bank (ID. 1ab4) (Morais Cabral et al. 1997). Molecular docking of ciprofloxacin with the

Table 1 Primer sequences used for the study

Name of the gene	Primer name	Primer sequence (5′ to 3′)	Size of the amplified product (bp)
gyrA	*gyrA* F	5′ TACACCGGTCAACATTGAGG 3′	648
	gyrA R	5′ TTAATGATTGCCGCCGTCGG 3′	
gyrB	*gyrB* F	5′ CAGACTGCCAGGAACGCGAT 3′	204
	gyrB R	5′ AGCCAAGCGCGGTGATAAGC 3′	
qnr	*qnr* F	5′ GATAAAGTTTTTCAGCAAGAGG 3′	543
	qnr R	5′ ATCCAGATCGGCAAAGGTTA 3′	

structure 1ab4 is performed using the PatchDock web server (Schneidman-Duhovny et al. 2005). Docking of the ciprofloxacin in PatchDock with clustering RMSD 1.5 is performed. 10 best solutions generated by the server are assessed based on their scoring and atomic contact energy and the complex is visualized by the Accelrys software package Discovery Studio Visualizer 2.0 (Accelrys, San Diego, CA).

Results

Hospital wastewater pollute environment with multi-drug resistant bacteria

Of the five groups of wastewater samples, the highest bacterial count was observed in sample 1 (Table 2), estimated 3.5 times higher than hospital non-connected sample (control 2), and the count was found decreasing with increasing distances of collection sites from waste disposal sites (samples 2 and 3, and control 1). Total resistant bacterial count was enumerated using ciprofloxacin as a selection marker due to its overuse by practitioners (Supplementary table 1). The estimation of resistant bacterial count at a drug concentration of 50 μg/mL, in sample 1 was found about 5-log higher than control 2, and the trends of resistance magnitude for other samples (2, 3 and control 1) over control 2 are also well comparable (Table 2). Besides, all of the 24 *E. coli* isolates were resistant to ten frequently-used antibiotics in CMCH, viz. ampicillin, bacitracin, cefalexin, ceftazidine, ciprofloxacin, cloxacillin, doxcyclin, novobiocin, sulphamethaxazole and vancomycin. None of the isolates were resistant to less frequently-used, or not used, antibiotics, viz. kanamycin, gentamycin, imipenem and nitrofurantoin (Supplementary table 1).

Plasmid profiling of ciprofloxacin resistant *E. coli*

Ten randomly selected isolates were taken out of 24 *E. coli* isolates, and these 10 isolates were resistant to ten most frequently-used antibiotics. Among these 10 isolates, four (ID 6, 16, 21 and 24) showed the same banding pattern containing multiple numbers of plasmid ranging from <2.0 to 110 MDa (Fig. 2). Middle ranged plasmids (3.1–3.9 MDa) were found to be present in two isolates (ID 8 and 18). Another two isolates (ID 20 and 23) showed one large plasmid and one middle ranged plasmid. Isolate ID 9 possesses both large and small-ranged plasmid. One isolate (ID 11), however, did not possess any plasmid.

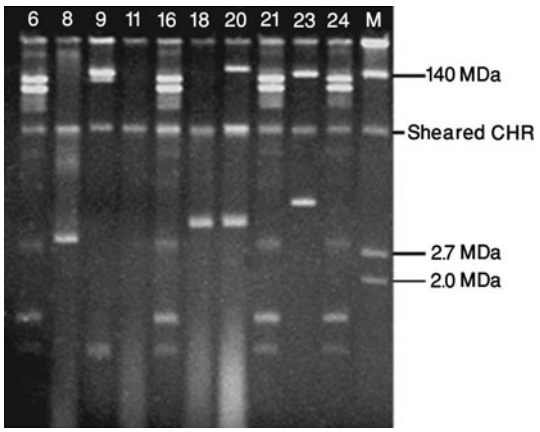

Fig. 2 Agarose gel electrophoresis of plasmid DNA of ten ciprofloxacin resistant *Escherichia coli* isolates. *E. coli* PDK-9 is used as a marker (*Lane-M*); CHR indicates the banding position of the chromosomal DNA

MBC_{100} of the resistant bacteria could not be attended

Based on the differences of plasmid profile, six resistant isolates (ID numbers 8, 9, 11, 16, 18 and 23) were selected for analyzing pattern of concentration-dependent resistance to ciprofloxacin using turbidimetric method. The bacterial population was found decreased with the increasing concentration of ciprofloxacin (Fig. 3). It may be mentioned that minimum bactericidal concentration (MBC_{100}) could not be determined for at least a sub-population of resistant bacteria, for they survived even at the highest concentration of ciprofloxacin used beyond of which the drug did not go in the solution.

Ciprofloxacin resistance occur due to mutation on QRDR region of DNA gyrase A

It was reported that the emergence of plasmid-mediated quinolone resistance are due to the presence of the *qnr*

Table 2 Hospital wastewater contained extremely high level of resistant bacteria compared to student dormitory wastewater control

Sample group	Total viable count/mL	Total viable count over control 2	Total resistant bacterial count/mL	Total resistant count over control 2	Percentage of resistance
1	1.136×10^8	3.55	1.54×10^7	1.57×10^5	13.54
2	5.20×10^7	1.6	7.52×10^4	7.67×10^2	0.15
3	1.41×10^7	0.44	8.20×10^3	83.67	0.06
4 (or, control 1)	9.20×10^6	0.28	9.33×10^3	95.20	0.10
5 (or, control 2)	3.20×10^7	1	9.80×10	1	0.0003

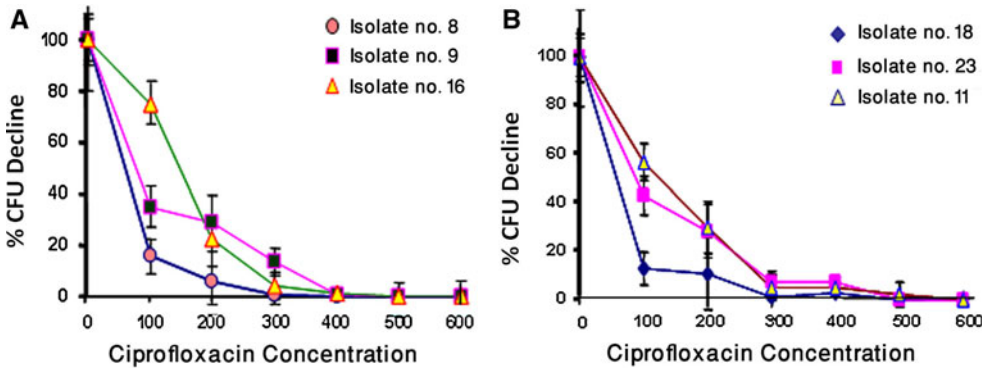

Fig. 3 Determination of the percent colony forming unit (CFU) decline with increasing concentration of ciprofloxacin (μg/mL) of samples 8, 9, 16 (**a**) and 18, 23, 11 (**b**)

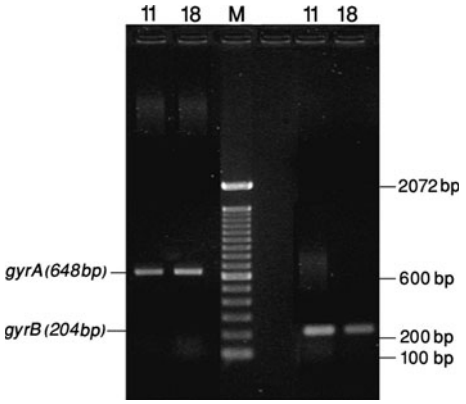

Fig. 4 Agarose gel electrophoresis of PCR amplification products of *gyr*A and *gyr*B gene. M-100 bp DNA molecular marker; Samples 11 and 18 are positive for *gyr*A (*left*) and *gyr*B (*right*)

genes whose products are capable of protecting DNA gyrase from quinolones (Mammeri et al. 2004; Robicsek et al. 2006). We attempted to amplify the *qnr* genes in the plasmid of the resistant *E. coli* isolates. However, no amplification was observed following the PCR reaction indicating the absence of *qnr* gene, and hence was concluded that the resistance to the drug was not plasmid-borne. This finding prompted us to look into *gyrA* and *gyrB* for the presence of mutation, if any. Two isolates were selected based on their plasmid profile, one had no plasmid (*E. coli* 11), and one had middle ranged plasmid (*E. coli* 18). A 648-bp amplified product of *gyrA* gene and a 204-bp amplified product of *gyrB* gene were detected in both of the isolates (Fig. 4).

Alterations in three amino acids were detected in the quinolone resistance determining region (QRDR) of GyrA subunit through sequencing. In both of the strains of *E. coli* (11 and 18), the mutations—S83L and D87Y were common, in addition to a different third change: A201S and A136 V for *E. coli* strains 11 and 18, respectively. On the other hand, no mutation was detected in the *gyr*B gene sequence.

For further understanding about how acquisition of mutation confers ciprofloxacin resistance, we modeled the interaction of ciprofloxacin with GyrA by using the already reported crystal structure of 59 kDa fragment of *E. coli* DNA gyrase subunit A (Fig. 5) (Morais Cabral et al. 1997). A low energy complex of −119 kJ/mol, geometric shape complementarity score of 3,826 and approximate interface area of complex 429.60 are accepted for further analysis. Residues making hydrophobic contact with the ciprofloxacin in the binding pocket are Ala 117, Gly 114, Ser 111 and Val 90 (Fig. 5a, b). Hydrogen bonding is formed between 4—oxo-group of ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-pyperazinyl)-3quinolinecarboxylic acid) and NH-group of Arg 91 (Fig. 5a). The presence of Arg 91 in this site appears very significant, as it stabilizes the drug-protein putative binding pocket through hydrogen bonding in one hand, and making salt-bridge interaction with Asp 87 on the other. This bridge is likely to cover the putative drug-binding slot, and thereby could stabilize the drug-binding complex. Mutation of D87Y would be expected to destabilize this core set of interactions. Figure 5b shows the electrostatic potential around the binding pocket of ciprofloxacin. Apart from the destruction of salt-bridge interaction, the mutation may be responsible for alteration of the electrostatic potential around the binding pocket.

Discussion

Most of the antibiotics (75–95%) used in healthcare, pharmaceuticals and households are expelled out of the body as partial- or un-metabolized form and are discharged into hospital sewage system. In Bangladesh, particularly, they are discharged directly into the municipal wastewater sewage system. The annual consumption of quinolones antibiotics, of which ciprofloxacin is the major one in CMCH wards is ~364 kg. The amount is calculated from the data provided by CMCH (500–1,500 mg/bed/day, personal communication). Considering the excretion rate of ciprofloxacin, ~273 kg of this single antibiotic is expelled in the Chittagong metropolitan area, thereby, contributing

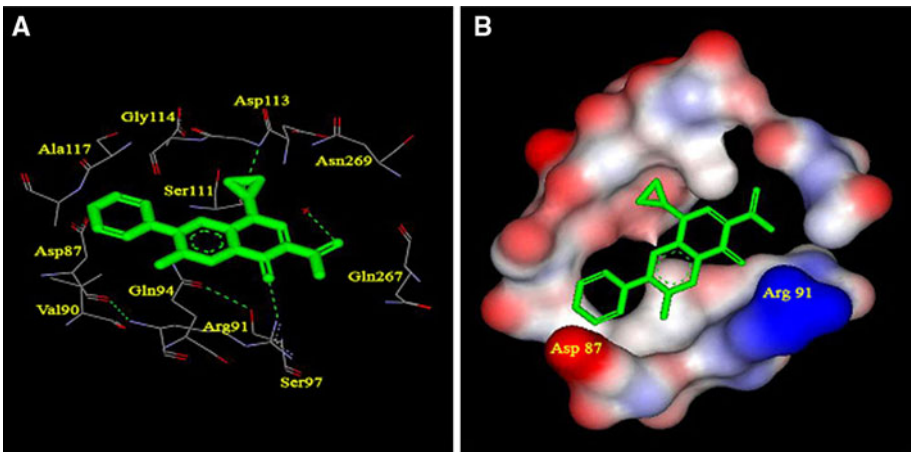

Fig. 5 Binding mode of Ciprofloxacin with DNA gyrase A subunit. **a** Asp 87 and Arg 91 forms salt bridge in the binding pocket. Arg 91 is directly involved in H-bonding interaction with ciprofloxacin. **b** Surface electrostatic potential around the binding pocket

to the environmental pollution. This finding corroborates with a study that measured about 0.7–124.5 mg/L of ciprofloxacin and 20–80 mg/L of β-lactams found in hospital effluents of a German hospital (Kummerer 2003). Besides, our recent work on Dhaka Medical College Hospital (DMCH) indicates that DMCH wastewater contained very high amount of ciprofloxacin and flucloxacillin too (Unpublished data).

Treatment of microbes with antibiotics may favor the growth of sub-fractions of populations that possesses the resistant properties. Our results clearly demonstrated that hospital wastewater contained ~5-log higher ciprofloxacin resistant bacteria, though the total bacterial count is 3.5 folds higher compared to the student dormitory control (Table 2). Further, the municipality drainage that carries mixed hospital- and non-hospital wastewater contain more than 200 times ciprofloxacin resistant bacteria but the total bacterial count was only 0.44 times of the student dormitory control. Thus, the results demonstrated that CMCH wastewater contaminate Chittagong metropolitan environmental components with multi-drug resistant bacteria (Table 2; Fig. 3). Similar observations and suggestions are also reported in other findings (Blondeau and Yaschuk 1996; Acar and Goldstein 1997).

If antibiotics select the sub-population resistant bacteria, one should expect higher percentage of resistant bacteria in our CMCH wastewater, whereas we have observed only 13.54% resistant bacteria. The results appear to be convincing under our experimental conditions, because the total resistant bacterial count was done in presence of higher concentration of ciprofloxacin (50 μg/mL), a much higher MIC_{50} value of ciprofloxacin since the sensitive and resistant level of ciprofloxacin is $\leq$1.0 μg/mL (Ednie et al. 1998) and $\geq$4.0 μg/mL, respectively (Muder et al. 1991).

All the *E. coli* isolates examined in the present study showed multi-drug resistance patterns which correlate with the antibiotics used by the practitioners to treat the patients (Supplementary table 1). The sensitivity pattern also showed that these isolates were sensitive to those antibiotics that were not used by the practitioners or that have limited use in public health. These findings can be explained that the used antibiotics and antibiotics contaminants present in hospital wastewater selectively favor the growth of the sub-population of the resistant bacteria. Similar presence of multi-drug resistant *E. coli* in sewage of hospital connection had been documented (Reinthaler et al. 2003).

Both chromosomal and plasmid born resistance to ciprofloxacin in the microorganisms have already been reported (Zhao et al. 1997; Tran and Jacoby 2002; Robicsek et al. 2006), and its spread either vertically or horizontally among the environmental bacteria is also reported earlier (Kasuya 1964). In Gram negative organisms, resistance to ciprofloxacin has been shown to be associated most frequently with alterations in gyr*A*. It has been found that the mutations are localized at the 5′ end of the gene (nucleotides 199 to 318 in the *E. coli gyrA*) in an area designated as the quinolone resistance-determining region (QRDR). A single mutation in codon 83 of *gyr*A was associated with decreased susceptibility or low levels of resistance to ciprofloxacin, and double mutations (codons 83 and 87) were associated with high levels of resistance (Weigel et al. 1998). As our *E. coli* isolates were highly resistant to ciprofloxacin (MIC_{50} values <150 μg/mL, Fig. 3), we hypothesized that more than one amino acid substitutions might be involved in QRDR in GyrA subunit. Our sequencing data confirmed that in addition to the S83L and D87Y, isolate numbers 11 and 18 possess a third mutation in the QRDR region, although their implication has not been characterized in this study.

The present study predicted the binding mode of ciprofloxacin with GyrA crystal structure using molecular docking approach (Morais Cabral et al. 1997). We have shown that Asp 87 forms salt bridge with Arg 91 in the putative ciprofloxacin binding pocket which confers its importance in the binding of ciprofloxacin. Previous work

(Barnard and Maxwell 2001) suggests that mutation in the S83A is largely without effect on enzyme activity, whereas mutation of D87 leads to loss of supercoiling activity, which can be attributed to stabilization of enzyme-DNA interactions. They also suggested that mutation of either residue may lead to the loss of enzyme-drug interactions. But importance of these residues in binding mode of ciprofloxacin was not clearly defined. From our observation, we can predict why mutation at D87 is destructive. Surface electrostatic potential of the binding pocket is also commensurate with our prediction. Mutation at position Asp 87 to hydrophobic Tyr (D87Y) probably leads to alteration of electrostatic potential in the binding pocket. Furthermore, higher ciprofloxacin resistance in *E. coli* was reported due to acquisition of double mutations in *gyrA* together with a mutation in efflux system (Olofsson et al. 2007). Therefore, we cannot rule out that the *E. coli* isolates might possess additional mutation in efflux genes *marR* or *acrR* or in topoisomerase *parC* and *parE* gene. Further work is needed for conclusive understanding of this mechanism.

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Conflict of interest Authors declared no conflict of interests.

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