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Characterization of Plasmid-mediated Quinolone Resistance within Clinical Isolates of Enterobacteriaceae: Study from Northeast India

Nargis Alom Choudhury[1], Debadatta Dhar (Chanda)[2], Nivedita Das Gupta[1], Anand Prakash Maurya[1], Atanu Chakravarty[2] and Amitabha Bhattacharjee[1]

[1]Department of Microbiology, Assam University, Silchar, India.
[2]Department of Microbiology, Silchar Medical College and Hospital, Silchar, India.

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The plasmid-mediated quinolone resistance gene (qnr) encodes a pentapeptide repeat protein that protects type II topoisomerase (DNA gyrase and topoisomerase IV) from quinolone antibiotics, resulting in resistance which is readily transmissible. Plasmid-mediated quinolone resistance (qnr) genes confer low-level resistance but provide background for selection of highly-resistant strains. We investigated quinolone resistance within clinical isolates of enterobacteriaceae and plasmids responsible for their transfer. A prospective, hospital-based study was conducted (September 2013 to march 2014). A total of 245 Consecutive, non-duplicate members of enterobacteriaceae were collected and subjected to screening of qnr resistant determinants, qnr genes detection using multiplex PCR, incompatibility typing and transferability assay was performed. Among 245 clinical isolates, 167 were screened to be resistant towards quinolone group of antibiotics. The quinolone resistance determinant was found to be transferable when transformants were selected in ciprofloxacin (.25μg/ml) screen agar. F inc type (n= 58) was more predominant, followed by K/B (n=40), Y (n=13), I (n=17) and P (n=7) in *E.coli* and I (n=6), K/B (n=7) in *Klebsiella* isolates, and K/B (n=6) in case of *Proteus* isolates when plasmids were typed by PCR based replicon typing. Ciprofloxacin resistance was lost after 48th consecutive serial passage. . The study calls for urgent steps to prevent over the counts availability of quinolone drugs, self medication and routine epidemiological investigation so as to minimize treatment failure due to quinolone therapy.

Keywords: *qnr*, ciprofloxacin, DNA gyrase, PMQR (plasmid mediated quinolone resistance).

Escherichia coli, *Klebsiella pneumonia* is an important nosocomial pathogen affecting both immunocompetent and immunocompromised patients and are responsible for a considerable proportion of infections in patients in Intensive Care Units (ICUs) worldwide. In last few decades, multidrug resistance in clinically dominant gram-negative bacilli is an emerging concern among health care practitioners worldwide, including often isolated members of Enterobacteriaceae and NFGNB (non fermenting gram negative bacilli).

Quinolone resistance in Enterobacteriaceae usually originates from mutations in target enzymes (DNA gyrase and/or topoisomerase IV) or because of impaired access to the target, occurring either because of changes in porin expression or because of over expression of efflux pumps. Recently, plasmid-mediated quinolone resistance (PMQR) has been detected conferring low-level resistance to quinolones by different mechanisms: a modifying enzyme [AAC (62)-Ib-cr]; the QepA and OqxAB efflux pumps; or gyrase and topoisomerase IV protection proteins

* To whom all correspondence should be addressed.
Mob.: +91-8471939088;
E-mail: ab0404@gmail.com

(Qnr). A unique allele has been described for the aac(62)-Ib-cr and oqxAB genes, two alleles have been described for qepA and several qnr genes, named qnrB, qnrS, qnrC and qnrD, have been described in various Gram-negative bacterial species since the first description of qnrA. Till date, 7 variants of QnrA (QnrA1 to QnrA7), 25 variants of QnrB (QnrB1 to QnrB25), 1 variant of QnrC, 4 variants of QnrS (QnrS1 to QnrS4) and 1 variant of QnrD are known. Although currently qnr-positive strains are relatively infrequent, widespread horizontal transfer among strains could contribute to the eventual compromise of the use of fluoroquinolones. Besides, qnr-positive strains in the absence of other resistance mechanisms are susceptible to fluoroquinolones according to the CLSI breakpoints, making their detection difficult[1].

Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid, a fully synthetic agent with bactericidal effects on most Enterobacteriaceae at clinical concentrations. Thus, these agents entered into use endowed with two advantages over the bacteria. First, although organisms could develop mutations that reduced quinolone susceptibility, the potency of these agents was such that a wild-type *Escherichia coli* would need to acquire spontaneously two or more resistance mutations to survive at clinical drug concentrations. So independent mutations generally arise once per 107 cell divisions or less, the likelihood that multiple mutations would occur in a single clone seemed negligible. Second, many resistance genes have co-evolved in nature with the antibiotics that they counteract, especially those that modify or inactivate the drug. Since the quinolones are fully synthetic, it seemed unlikely that resistance genes would be available for recruitment onto mobile elements. Thus, the quinolones seemed to confound resistance; they were a class of agents to which mutational resistance was unlikely to develop and against which resistance genes could not be acquired[2].

Therefore, the present study was undertaken to investigate quinolone resistance within clinical isolates of enterobacteriaceae and plasmids responsible for their transfer.

Methodology

Sample size

A total number of 245 consecutive, non duplicate members of enterobacteriaceae family were collected during a period of six months from september 2013 to march 2014 from clinical specimens from hospital patients who were admitted or attended OPDs of Silchar Medical College and Hospital. Isolates were identified based on cultural characteristics and conventional biochemical testing methods.

Antibiotic susceptibility and phenotypic screening of quinolone resistance

Phenotypic screening of quinolones was performed by Kirby bauer disc diffusion method using following antibiotics (Hi-Media, Mumbai, India): nalidixic acid (30µg), norfloxacin (10µg), ofloxacin (5µg) and ciprofloxacin (10µg) additionally susceptibility was determined against gentamicin (10µg), amikacin (30µg), meropenem (10µg), cotrimoxazole (25µg). *E. coli* ATCC 25922 was used as quality control strain. Results were interpreted according to CLSI guidelines[3].

Determination of Minimum Inhibitory Concentration (MIC): MIC was determined by agar dilution method on MHA (Muellar Hinton Agar) against norfloxacin, ciprofloxacin and ofloxacin and results were interpreted according to CLSI breakpoint[3].

Genotypic characterization of quinolone resistance

Multiplex PCR assay was performed targeting *qnr A, qnr B, qnr S* and *qep A.* Primers used in this study was as described earlier[4].

The amplification was performed on 25µl of total volume containing 1.5µl of template DNA, 10pm of each primer, and 12.5µl of Go green Taq polymerase (Promega, Madison, USA). PCR conditions were used as following: denaturation at 95°C for 2 min, 32 cycles with denaturation at 95°C for 25 sec, annealing at 52°C for 45 sec and extention at 72°C for 1 min, and final extension was performed at 72°C for 5 min. PCR products were separated by electrophoresis on 1% agarose gel and visualized using a gel documentation system after staining with ethidium bromide.

Transferability of quinolone resistance

Isolated plasmids were tested for their ability of horizontal transfer by transformation assay. The recipient strain used was *E.coli* JM107. Transformation was carried out by heat shock method[5]. Transformants were selected on LB Agar plates containing ciprofloxacin (0.25µg/ml).

Analysis of stability of plasmid encoding quinolone resistance gene

The experiment was performed to determine the vertical transfer of resistant genes along with their plasmids in subsequent generations. Plasmid stability of screened quinolone resistant isolates as well as their transformants were analyzed by serial passages method for consecutive 75 days at 1:1000 dilutions without any antibiotic pressure[6].

Determination of plasmid incompatibility typing

Plasmid incompatibility was determined for wild type as well as transformants by PCR based replicon typing using 18 pairs of different basic replicon primers, targetting FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons[7].

Typing of isolates

DNA fingerprinting by *enterobacteriaceae repetitive intergenic consensus sequence (*ERIC*)* PCR[8] was performed for quinolone resistant gene. The primers used for ERIC-PCR were as follows: ERIC-1R (5'-AAGCTCCTGGGGATTCA-3') and ERIC-2F (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Each PCR amplification was performed on 25 µl of total volume containing 1.5 µl of template DNA, 10pm of each primer, and 12.5µl of Go green Taq mixture (Promega, Madison, USA). PCR conditions were used as following: denaturation at 95°C for 2 min, 32 cycles with denaturation at 95°C for 25 sec, annealing at 52°C for 45 sec and extention at 72°C for 1 min and final extension was performed at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized using a gel documentation system after staining with ethidium bromide.

RESULTS

Sample size

Out of 245 isolates167 organisms were found resistant; among them the most predominant organism was found were *Escherichia coli* (n=121) followed by *Klebsiella pneumoniae* (n=25) and *Proteus* spp. (n=21). All the isolates showed multi-drug resistance (MDR) phenotypes (table 1).

Table 1.

Organism	No. of isolate
Escherichia coli	121
Klebsiella pneumonia	25
Proteus spp.	21

Table 2. Table showing different antibiogram

	Gentam-icin	ciprofloxac in	Cotrimo-xazole	amikacin	imepenem	Nalidixic acid	ofloxac in	Norfloxa-cin
E.coli	121	121	121	121	121	121	121	121
K. pneumoniae	25	25	25	25	25	25	25	25
Proteus spp.	21	21	21	21	21	21	21	21

Susceptibility and phenotypic screening of quinolone resistant organisms

The MIC of the isolate was shown in table 2 and 3 (a,b & c). It was found that majority (n=121) of the *Escherichia coli* spp. isolates were above the breakpoint for all the drugs tested.

Genotypic characterization of quinolone resistance

PCR results established the presence of plasmid-mediated quinolone resistance in the isolates. The plasmid mediated resistant determinants, *qnrA*, *qnrB*, *qnrS*, and qepA were detected by PCR.

Transferability of quinolone resistance

Isolated plasmids were tested for their ability of horizontal transfer by transformation assay. The recipient strain used was *E.coli* JM107. Transformation was carried out by heat shock method. Transformants were selected on LB Agar plates containing ciprofloxacin (0.25µg/ml). It was observed that transformants showed ciprofloxacin resistance (for *E.coli* (n=67), *for Klebsiella pneumonia* (n=6), and for *Proteus spp.* (n=3).

Analysis of stability of plasmid encoding quinolone resistance gene

Transformants retained resistance till 70th passage ciprofloxacin was found to have moderate

Table 3(a). Showing MIC against ciprofloxacin

Organism/concentration of antibiotic	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml
E.coli	106	5	3	3	7
Klebsiella pneumoniae	20	-	-	-	5
Proteus spp.	16	5	-	-	-

Table 3(b). Showing MIC against norfloxacin

Organism/concentration of antibiotic	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml
E.coli	89	17	7	-	8
Klebsiella pneumoniae	16	-	-	14	-
Proteus spp.	14	-	7	-	-

Table 3(c). Showing MIC against ofloxacin

Organism/concentration of antibiotic	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml
E.coli	75	-	23	-	23
Klebsiella pneumoniae	15	-	-	15	-
Proteus spp.	13	-	-	-	8

Table 4. Total number of quinolone-resistance isolates (*n*=167)

Number of quinolone-resistance isolates	*qnrS*	*qnrA*	*qnrB*	*qepA*
E.coli (n=121)	3	18	0	11
Klebsiella pneumonia (n=30)	1	3	0	3
Proteus spp. (n=21)	2	5	0	0

activity in stability after 48th number of serial passages.

Determination of plasmid incompatibility typing

All the transformants were subjected to PCR based replicon typing (5 multiplex and 3 simplex PCR), and it was observed that F inc (n=58) type was more prevalent, followed by K/B (n=40) and Y (n=13), I (n=17) and P (n=7) inc type was also observed in *E.coli* isolates. Whereas, I (n=6) and K/B (n=7) inc type was found in *Klebsiella* isolates and K/B (n=6) inc type was found in *Proteus* isolates.

DNA fingerprinting

ERIC PCR revealed that three different clonal types were found in the isolates of *E.coli* and others are non-related for *Klebsiella* and *Proteus.*

DISCUSSION

Fluoroquinolones are broad-spectrum and highly bactericidal antimicrobials agents that are used to treat various bacterial infection. *Escherichia coli* infections, especially urinary tract interactions, are frequently treated with fluoroquinolones, and fluoroquinolone resistance has increased in the clinical field. Fluoroquinolone resistance is caused mainly by chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, which encode DNA gyrase subunits, and *parC* and *parE*, which encode topoisomerase IV subunits[9]. In other gramnegative bacteria, such as *Escherichia coli*, *Neisseria gonorrhoeae*, or *Klebsiella pneumoniae*, high-level fluoroquinolone resistance is always associated with the presence of multiple mutations

in the quinolone resistance-determining regions (QRDRs) of the genes that code for the intracellular targets of these antibiotics, gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*)[10].

However, plasmid mediated quinolone resistance also contributes a major factor within hospital and community acquired infection. In our study, it is clearly established that qnrA and qepA had predominantly involved in lateral transfer of quinolone resistance. Previous Indian studies have reported phenotypic quinolone resistance (11). But the molecular background of the resistance is lacking. Presence of diverse incompatible types carrying quinolone resistance signifies their multiple source of acquisition and origin within the study area. The study has also underscores polyclonal spread of quinolone resistant organism in their area which is supported by ERIC PCR data. Conclusion: The study calls for urgent steps to prevent over the counters availability of quinolone drugs, self medication and routine epidemiological investigation so as to minimize treatment failure due to quinolone therapy.

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