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Plasmid-mediated fluoroquinolone resistance associated with extra-intestinal *Escherichia coli* isolates from hospital samples

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***Background & objectives*: Infection from fluoroquinolone-resistant extra-intestinal *Escherichia coli* is a global concern. In this study, isolation and characterization of fluoroquinolone-resistant extra-intestinal *E. coli* isolates obtained from hospital samples were undertaken to detect plasmid-mediated quinolone resistance (*PMQR*) genes.**

***Methods*: Forty three isolates of *E. coli* obtained from patients with extra-intestinal infections were subjected to antibiogram to detect fluoroquinolone resistance. The mechanism of fluoroquinolone resistance was determined by the detection of PMQR genes and mutations in quinolone resistance determining region (QRDR).**

***Results*: Of the 43 isolates, 36 were resistant to nalidixic acid (83.72%) and 28 to ciprofloxacin (65.11%). Eight *E. coli* isolates showed total resistance to both the antimicrobials without any minimum inhibitory concentration. The detection of PMQR genes with *qnr* primers showed the presence of *qnrA* in two, *qnrB* in six and *qnrS* in 21 isolates. The gene coding for quinolone efflux pump (*qepA*) was not detected in any of the isolates tested. The presence of some unexpressed PMQR genes in fluoroquinolone sensitive isolates was also observed.**

***Interpretation & conclusions*: The detection of silent PMQR genes as observed in the present study presents a risk of the transfer of the silent resistance genes to other microorganisms if present in conjugative plasmids, thus posing a therapeutic challenge to the physicians. Hence, frequent monitoring is to be done for all resistance determinants.**

Key words Antibiotic resistance - plasmid-mediated quinolone resistances - quinolone resistance determining regions

The increasing trend of antibiotic resistance among bacterial pathogens is a cause of global concern[1]. *Escherichia coli,* a member of the family *Enterobacteriaceae*, is known to cause extra-intestinal infections frequently showing resistance to fluoroquinolones[2]. Although a normal flora in the intestinal tract of human and animals, pathogenic strains of *E. coli* cause intestinal infections such as gastroenteritis and extra-intestinal conditions such as urinary tract infection (UTI), meningitis, septicaemia,

nosocomial pneumonia, osteomyelitis and wound infections. Virulence factors, such as adhesins and exotoxins, play an important role in the pathogenesis of this microorganism[3]. Although the major reservoir of extra-intestinal pathogenic *E. coli* (ExPEC) causing infection remains the alimentary tract, other sources, such as contaminated food, are also incriminated[4]. ExPEC is known to harbour specialized virulence factors to cause extra-intestinal disease particularly UTI infections[5,6]. Such isolates can pose a significant threat to public health since these can also harbour resistance determinants that can make a pathogen resistant to multiple antimicrobial classes including present generation cephalosporins and fluoroquinolones[7].

Fluoroquinolones are an effective class of drugs used by clinicians for treating the infections of Gram-negative pathogens including UTI and hospital-acquired infections. However, the indiscriminate use of these antimicrobials has increased the prevalence of quinolone and fluoroquinolone resistance in bacterial pathogens usually mediated by point mutations in topoisomerase II (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes, as well as by the overexpression of efflux pumps[8]. In addition, plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrS*, *qnrD, qnrE* and *qnrVC*), associated with a modified aminoglycoside acetyltransferase gene [*aac* (6′)-1b- cr] and a specific quinolone efflux pump *qepA* and *oqxAB* have also been described in *Enterobacteriaceae*[2,9,10]. The PMQR genes in bacteria are known to display reduced susceptibility to fluoroquinolones; however, these may not present mutations in quinolone resistance determining region (QRDR)[11].

Plasmid-mediated quinolone resistance mechanism is a cause of concern since PMQR genes are located on conjugative plasmids and have been shown to disseminate fluoroquinolone resistance in *E. coli* isolates. The pentapeptide protein complex encoded by *qnr* determinants are thought to bind to topoisomerase II preventing it from the action of fluoroquinolones, *aac*(6')-lb-cr is known to modify fluoroquinolones with piperazinyl moiety, while plasmid-mediated *qep*A encodes an efflux pump of major facilitator family[12].

Material & Methods

Bacterial strains & determination of antimicrobial susceptibility testing: Phenotypic identification of *E. coli* isolates (n=43) from clinical samples obtained from Madras Medical Mission, Department of Microbiology, Mogappair and Chennai, India, over the period of two years (2015-2017) was done and these isolates were subjected to antibiotic sensitivity testing. The isolates were analyzed for quinolone/fluoroquinolone susceptibility using disc diffusion assay according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[13]. Briefly, the bacterial isolates with the density of 0.5 McFarland turbidity were swabbed onto the pre-poured and dried Mueller Hinton agar (HiMedia Laboratories Pvt. Ltd., Mumbai). The antibiotic discs of ciprofloxacin (CIP) (5 µg) and nalidixic acid (NA) (30 µg) (HiMedia) were placed on the bacterial lawn using a sterile applicator. After overnight incubation at 37°C, inhibition zone diameters were measured and interpreted as resistant, sensitive or intermediate sensitive as per CLSI guidelines[13]. *E. coli* (ATCC 25922) was used as a quality control strain.

Characterization of PMQR genes: All the 43 isolates were checked for genus specific gene and for the presence of PMQR genes using PCR[14,15]. PCR was performed in 30 µl reaction volumes containing 3 µl of 10 × buffer [100 mM Tris-HCl (*p*H 9), 1.5 mM $MgCl_2$, 50 mM KCl and 1% gelatine], 100 µM of four deoxyribonucleotide triphosphates each (dATP, dGTP, dCTP and dTTP), 10 pmol of each forward and reverse primers and 1.0 U of *Taq* DNA polymerase with 2 µl of template DNA. All the isolates were tested for the presence of PMQR genes using primers listed in the Table I. The amplified PCR products were further purified using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany).

Detection of mutation in QRDR using mismatch amplification mutation-MAMA-PCR: A MAMA assay was performed on the 43 isolates to detect the point mutations in the QRDR[16]. The known point mutations at amino acid position 83 and 87 of *gyr*A, 80 and 84 of *par*C, 447 of *gyr*B and 416 amino acid position of *parE* were targeted[17]. The primers used in the study are outlined in Table I.

PCR was performed in 30 µl reaction volumes containing 3 µl of 10X *Taq* buffer, 83 µM of four deoxyribonucleotide triphosphates, 30, 20 and 10 picomoles of forward, MAMA reverse and control reverse primers, respectively and 1 U of *Taq* DNA polymerase with 2 µl of DNA as a template. PCR amplification was carried out in a thermal cycler (Bio-Rad, USA) with the initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C,

Table I. Oligonucleotide primers used for determining gyrase and topoisomerase IV target genes

Primers	Sequence (5'-3')	Annealing temperature	References
	Genus specific primer		
uidA	F-AAAACGGCAAGAAAAAGCAG R-ACGCGTGGTTACAGTCTTGCG	63ºC	14
	QRDR mutation detection primers		
gyrA	F-GAC CTT GCG AGA GAA ATT ACA C R-GAT GTT GGT TGC CAT ACC TAC G	55°C	16
parC	F-CGG AAA ACG CCT ACT TAA ACT A R-GTG CCG TTA AGC AAA ATG T	55°C	16
MAMA *gyrA*83	R-TCG TGT CAT AGA CCG GGC	55°C	16
MAMA *gyrA*87	R-GCG CCA TGC GGA CGA TCG TTT C	55°C	16
MAMA *parC*80	R-ATC GCT TCA TAA CAG GCT CT	55°C	16
MAMA *parC*84	R- CCA TCA GGA CCA TCG CCT C	55°C	16
gyrB	F-GTG AAA TGA CCC GCC GTA AA R-TGA TAA GCG TCG CCA CTT CC	55°C	17
ParE	F-TCT GGC CGG ATG AAA CCT TC R-TTT CAG TGG CAT GAT CGC CT	55°C	17
MAMA *gyrB* 447	R-GAC GTT GAG GAT TTT ACC CTC	55°C	17
MAMA *parE* 416	R-GCG GAG TCA CCT TCC ACT AG	55°C	17
	PMQR detection primers		
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	55°C	15
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	55°C	15
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	55°C	15
qepA	F: CGTGTTGCTGGAGTTCTTC R: CTGCAGGTACTGCGTCATG	59°C	15

PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance determining region; MAMA, mismatch amplification mutation assays

annealing at 55°C and extension at 72°C for 40 seconds respectively, and a final extension at 72°C for 10 min. PCR products were visualized on two per cent agarose gel stained with ethidium bromide (0.5 µg/ml) in 1× tris-acetate EDTA (TAE) buffer loaded with 10 µl of the reaction mixture and observed under UV light in a Gel Documentation system (Bio-Rad, USA).

DNA sequence analysis: The purified PCR products were sequenced in an automated ABI 3100 Genetic analyser (Applied Biosystems, USA) using fluorescent label dye terminators. The nucleotide sequences were analyzed using BLAST programmes, blastn and blastp (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*). The amino acids deduced from the DNA sequences were obtained through a web-based programme Expasy Translate tool (*https://web.expasy.org/translate/*) following which the novel sequences were submitted to the GenBank.

Results

Forty-three extra-intestinal isolates of *E. coli* obtained from biological samples from patients with UTIs (21), wound infections (6), neonatal meningitis (5), septicaemia (5), nosocomial pneumonia (4) and osteomyelitis (2) from all age groups confirmed by phenotypic tests were reconfirmed as *E. coli* by PCR-based genotypic test for *uid*A gene.

Antibiogram analysis: Of the 43 quinolone/fluoroquinolone-resistant isolates, 28 (65.11%) were found to be resistant to ciprofloxacin while 36 (83.72%) were resistant to nalidixic acid. Seven isolates were

sensitive to both the antibiotics and three showed intermediate sensitivity to ciprofloxacin.

Characterization of PMQR genes: Results of plasmid-mediated quinolone resistance genes identified by PCR using PMQR primers revealed 29 of the 43 isolates (67.44%) harbouring PMQR genes. Among these, two (6.89%), six (20.68%) and 21 (72.41%) were positive for *qnrA*, *qnrB* and *qnrS,* respectively (Fig. 1A and B). Seven (16.27%) isolates which were sensitive to both the antibiotics by phenotypic test, possessed plasmid-mediated quinolone resistance genes, one of which was (*qnrB*) sequenced and submitted to the GenBank.

Detection of mutations in QRDR using MAMA-PCR: The MAMA PCR in the absence of mutations(s) in sensitive isolates generated two PCR products from the wild-type gene using universal forward/reverse and MAMA reverse primers whereas, a single amplicon was produced in resistant isolates with QRDR mutation(s) due to the inhibition of PCR in the presence of two or more mismatches at the 3'end of the MAMA primer (Fig. 2A-D). Nine (20.9%) of the 43 isolates resistant to both the antimicrobial agents harboured mutation only at amino acid position 83 of *gyr*A while one of the isolates possessed mutation only at position 87 of the same gene. Thirteen isolates (30.23%) resistant to both the antibiotics presented mutations at *gyr*A 83, 87 and at *par*C 80 regions. Two (4.65%) of the isolates which were resistant to only nalidixic acid harboured mutations at both *gyr*A position 83 and 87. Four (9.30%) isolates showing resistance to both the antibiotics harboured mutation at amino acid position 83 of *gyr*A and 80 of *par*C while four (9.30%) isolates had mutation at position 83 of *gyr*A and 84 of *par*C. An isolate of *E. coli* resistant to both the antibiotics displayed mutation at all the four important positions of QRDR (*gyr*A 83, 87 and *par*C 80, 84) (Table II). An isolate resistant to nalidixic acid did not harbour any mutation in the QRDR. None of

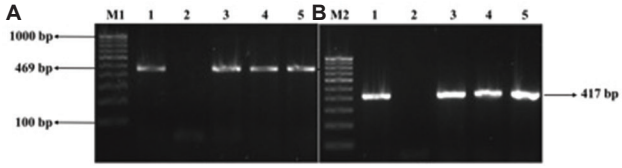

Fig. 1. Agarose gel electrophoresis image of PMQR genes (**A**) M1: 100 bp DNA ladder; lane 1: *qnrB* PCR product; lane 2: negative control; lanes 3-5: isolates positive for *qnrB*. (**B**) M2: 100 bp DNA ladder; lane 1: *qnr*S PCR product; lane 2: negative control; lanes 3-5: isolates positive for *qnrB*. PMQR, plasmid-mediated quinolone resistance.

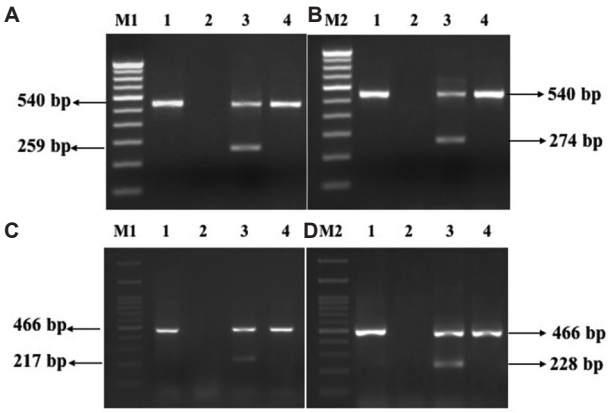

Fig. 2. PCR products of duplex MAMA-PCR assays (**A**) MAMA *gyrA* 83. M1: 100 bp DNA ladder; lane 1: *gyrA* PCR; lane 2: negative control; lane 3: *gyrA* 83 without mutation; lane 4: *gyrA* 83 with mutation. (**B**) MAMA *gyrA* 87. M2:100 bp DNA ladder; lane 1: *gyrA* PCR; lane 2: negative control; lane 3: *gyrA* 87 without mutation; lane 4: *gyrA* 87 with mutation. (**C**) MAMA *parC* 80. M1:100 bp DNA ladder; lane 1: *parC* PCR; lane 2: negative control; lane 3: *parC* 80 without a mutation; lane 4: *parC* 80 with a mutation. (**D**) MAMA *parC* 84. M2:100 bp DNA ladder; lane 1: *parC* PCR; lane 2: negative control; lane 3: *parC* 84 without mutation; lane 4: *parC* 84 without mutation. MAMA-PCR, mismatch amplification mutation assays-polymerase chain reaction.

Table II. Mutation status of *Escherichia coli* isolates at topoisomerase targets

Total number of mutations	Number (%) of isolates	Alterations detected by MAMA-PCR					
		gyrA 83	*gyrA* 87	*parC* 80	*parC* 84	*gyrB* 447	*parE* 416
1	9 (20.93)	Mutation	None	None	None	None	None
	1 (2.32)	None	Mutation	None	None	None	None
2	4 (9.30)	Mutation	None	Mutation	None	None	None
	2 (4.65)	Mutation	Mutation	None	None	None	None
	4 (9.30)	Mutation	None	None	Mutation	None	None
3	13 (30.23)	Mutation	Mutation	Mutation	None	None	None
4	1 (2.32)	Mutation	Mutation	Mutation	Mutation	None	None

MAMA, mismatch amplification mutation assays

the isolates showed mutation in the *gyr*B and *par*E regions. The PCR products of *gyr*A and *par*C of one of the representative *E. coli* isolate were sequenced and analyzed. The partial sequences with possible QRDR mutations were submitted to the GenBank and were assigned GenBank accession numbers MF288967 for *gyr*A and MF2889868 for *par*C.

Discussion

Fluoroquinolones are a major class of antimicrobial drugs used widely for the treatment of infections caused by Gram-negative bacterial pathogens. *E. coli* being one of the major causes of several extra-intestinal and hospital-acquired infections is recognized as a major problem to tackle as it shows resistance to most of the quinolones and fluoroquinolones. Mechanisms underlying fluoroquinolone resistance were earlier thought to be confined to vertical inheritance due to the spontaneous occurrence of point mutations in QRDR regions[18]. However, now it is reported to be spread horizontally using plasmid-mediated *qnr* genes and efflux pump genes[19]. Although, in most of the cases, fluoroquinolone resistance is attributed to mutations in the QRDR regions, a reasonable percentage of isolates in this study also harboured PMQR genes and is in agreement with the reports of Kao *et al*[20]. Among the PMQR positive isolates the highest percentage harboured *qnr*S (77.77%) followed by *qnr*B (22.22%) and *qnr*A (11.11%); however, *qep*A was not observed in any of the isolates. In contrast, a study from China[21] showed presence of *qnr*S in isolates followed by *qep*A with the absence of *qnr*A and *qnr*B. Another study from China[22] showed the presence of *qnr*, *aac(6')-Ib-cr*, *qep*A and *oqx*AB in 2.7, 24.5, 11.9 and 6.3 per cent, respectively of fluoroquinolone-resistant *E. coli* isolates. In a study from Korea, Yang *et al*[23] observed PMQR genes in 73.8 per cent of ciprofloxacin-resistant *E. coli* isolates. Although, in our study, the sample size was small, yet our results showed the presence of PMQR genes in 67.44 per cent of the *E. coli* isolates included. PMQRs, such as *qep*A and *aac-(6')-Ib,* were found to be dominant in the aquatic environments[24]. This observation supported the notion that the aquatic environment might constitute the original source of PMQR genes[25]. Our study also demonstrated that extra-intestinal *E. coli* isolates might carry silent antibiotic resistance genes, since a few fluoroquinolone sensitive isolates (16.27%) of *E. coli* harboured PMQRs such as *qnr*B and *qnr*S. Perhaps this gene did not express in these isolates as phenotypic resistance. The reason for the silent nature of the *qnr*S and *qnr*B of the sensitive isolates needs further study. Silencing of antibiotic resistance genes may be a phenomenon that has not received much attention. Enne *et al*[26] reported silencing of several plasmid-borne (pVE46) antibiotic resistance genes such as bla_{oxa-2}, *aadA1*, *sul1* and *tetA* in *E. coli* isolated from pig following oral inoculation of organic piglets. There was no deletion of genes or promoter regions. However, the silent resistance genes were expressed again when the plasmid carrying resistance genes transferred to a new host. This suggested that the silencing phenomenon was due to the chromosomal effects of the host. Later, it was also found that the silencing was reversed at a low frequency of 10^{-6}-10^{-10} in the original host[26]. Deekshit *et al*[27] suggested that the deletion of promoter region was the main reason for unexpressive nature of the chloramphenicol acetyltransferase (*catA*) gene in *Salmonella* Weltevreden. These reports show the future risk associated with the global emergence of plasmid-borne resistance pattern of clinical pathogens and these PMQRs can also contribute to the elevated levels of ciprofloxacin minimum inhibitory concentrations (MICs) in clinical isolates.

All the fluoroquinolone-resistant isolates harbouring PMQR genes also had QRDR mutations. A MAMA-PCR was used to detect mutations at four major QRDRs (*gyr*A 83, *gyr*A 87, *par*C 80 and *par*C 84). It has been used to detect point mutations in *gyr*A and *par*C regions in fluoroquinolone-resistant bacterial pathogens[28-30]. The point mutation S83L at *gyr*A 83 was the most commonly observed change[31,32] followed by mutation at *gyr*A 87. In the present study, 76.44 per cent (33/43) of the isolates harboured point mutation at *gyr*A 83 and 39.53 per cent (17/43) at *gyr*A 87. Thus, it is important to analyze these regions to check the multiple mutation status of resistant pathogens.

Among the 43 isolates of *E. coli*, seven different patterns of QRDR mutations were observed. Although, in the present study, all the mutations at *gyr*A 87 were associated with mutation at *gyr*A 83, one nalidixic acid-resistant isolate (S5) harboured mutation only at *gyr*A position 87. Higher levels of quinolone/fluoroquinolone resistance with increased MICs in many bacterial pathogens are usually associated with double mutations in *gyr*A region[33]. Similarly, in our study, the isolates resistant to both the antibiotics harboured double mutation at *gyr*A and other regions. Some of the nalidixic acid-resistant isolates did not show any mutation in the QRDRs as reported by us earlier also[34]. Since PMQRs

are mainly known to increase the MIC in resistant isolates, its exact relevance as a sole mechanism of fluoroquinolone resistance needs further study.

In conclusion, our study showed the occurrence of plasmid-mediated resistance as the second most encountered fluoroquinolone resistance mechanism among clinical isolates of *E. coli*. In addition, the detection of silent PMQR genes in sensitive isolates could pose a future risk of these isolates transforming into resistant forms upon antibiotic challenge. Hence, it is advisable to check for the presence of resistance determinants even in phenotypic sensitive isolates.

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***Conflicts of Interest*:** None.

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