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QRDR mutations, efflux system & antimicrobial resistance genes in enterotoxigenic *Escherichia coli* isolated from an outbreak of diarrhoea in Ahmedabad, India

G.P. Pazhani, S. Chakraborty, K. Fujihara*, S. Yamasaki*, A. Ghosh, G.B. Nair & T. Ramamurthy

National Institute of Cholera & Enteric Diseases (ICMR) & Kolkata, India & *Graduate School of Agriculture & Biological Sciences, Osaka Prefecture University, Osaka, Japan

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Background & objectives: Diverse mechanisms have been identified in enteric bacteria for their adaptation and survival against multiple classes of antimicrobial agents. Resistance of bacteria to the most effective fluoroquinolones have increasingly been reported in many countries. We have identified that most of the enterotoxigenic *Escherichia coli* (ETEC) were resistant to several antimicrobials in a diarrhoea outbreak at Ahmedabad during 2000. The present study was done to identify several genes responsible for antimicrobial resistance and mobile genetic elements in the ETEC strains.

Methods: Seventeen ETEC strains isolated from diarrhoeal patients were included in this study. The antimicrobial resistance was confirmed by conventional disc diffusion method. PCR and DNA sequencing were performed for the identification of mutation in the quinolone resistance-determining regions (QRDRs). Efflux pump was tested by inhibiting the proton-motive force. DNA hybridization assay was made for the detection of integrase genes and the resistance gene cassettes were identified by direct sequencing of the PCR amplicons.

Results: Majority of the ETEC had GyrA mutations at codons 83 and 87 and in ParC at codon 80. Six strains had an additional mutation in ParC at codon 108 and two had at position 84. Plasmid-borne *qnr* gene alleles that encode quinolone resistance were not detected but the newly described *aac(6')-Ib-cr* gene encoding a fluoroquinolne-modifying enzyme was detected in 64.7 per cent of the ETEC. Class 1 (*int11*) and class 2 (*int12*) integrons were detected in six (35.3%) and three (17.6%) strains, respectively. Four strains (23.5%) had both the classes of integrons. Sequence analysis revealed presence of *dfrA17*, *aadA1*, *aadA5* in class 1, and *dfrA1*, *sat1*, *aadA1* in class 2 integrons. In addition, the other resistance genes such as *tet* gene alleles (94.1%), *catAI* (70.6%), *strA* (58.8%), *bla_{TEM-1}* (35.2%), and *aphA1-Ia* (29.4%) were detected in most of the strains.

Interpretation & conclusions: Innate gene mutations and acquisition of multidrug resistance genes through mobile genetic elements might have contributed to the emergence of multidrug resistance (MDR) in ETEC. This study reinforces the necessity of utilizing molecular techniques in the epidemiological studies to understand the nature of resistance responsible for antimicrobial resistance in different species of pathogenic bacteria.

Key words Efflux system - ETEC - integrons - mutations - QRDR - resistance genes

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhoea due to their expression of either heat-labile (LT) or heat-stable (ST) or both the enterotoxins. In developing countries, ETEC is generally associated with sporadic infection especially among children and travelers¹. The first outbreak of ETEC mediated diarrhoea in India was reported from Ahmedabad, Gujarat, during 2000 and majority of the strains expressed multidrug resistance². In clinical practice, there is a great deal of speculation regarding therapeutic and subtherapeutic use of antimicrobials as it helps in accelerating the development and dissemination of antimicrobial-resistant bacterial pathogens¹.

Quinolones and fluoroquinolones are the firstline drugs for the treatment of diarrhoea for many years. However, several studies have documented the emergence and spread of fluoroquinolone resistant enteric pathogens in India³⁻⁶. Quinolone resistance mechanisms have been described and characterized in a variety of bacteria⁷. These have mostly been related to specific mutations that lead to amino acid alterations in the quinolone-resistance determining regions (QRDRs) within the subunits constituting topoisomerases II (GyrA and GyrB) and IV (ParC and ParE), which are involved in DNA replication, recombination, transcription and in the partitioning the replicated chromosome⁷. Most of the guinolone resistant clinical isolates of E. coli have mutations in the QRDRs of both the gyrA and the parC. In addition, a novel mechanism of plasmid-mediated quinolone resistance (PMQR) was reported that involves DNA gyrase protection by a protein from the pentapeptide repeat family called Qnr⁸. Qnr determinant confers resistance to nalidixic acid and increases the MICs of fluoroquinolones by 4-8 folds and supplements the resistance with other mechanisms8. Recent studies stress the importance of the PMQR among different pathogenic bacteria of clinical origin^{9,10}. The qnr gene alleles were also detected in bacterial strains that express extended spectrum β -lactamase (ESBL). In addition, involvement of many efflux pump mechanisms and presence of a novel gene, *aac(6')-Ib-cr* encoding a fluoroquinolne-modifying enzyme (aminoglycoside acetyltransferase) have been detected in many enteric pathogens¹¹.

Mobile genetic elements such as integrons have been identified in many multidrug resistant bacteria that play an important role in the acquisition and dissemination of several antibiotic resistance genes¹². To date, at least eight classes of integrons have been described and each class is distinguished by differences in the sequences of the integrase gene. Majority of integrons identified among clinical isolates belongs to class 1 type¹³. Class 2 integrons exists in the transposon Tn7 or its derivatives, and the 3'-CS contains five *tns* genes involved in the movement of the transposon¹⁴. More than 60 different antibiotic-resistance genes, covering most antimicrobials presently in use, have been characterized in cassette structures. In all the reported cases, the cassettes have been inserted in the same orientation and are usually transcribed from a promoter in the 5'-CS¹⁵.

In this study, 17 ETEC strains isolated from a diarrhoeal outbreak in Ahmedabad during 2000, were analysed for mutation in the QRDR of *gyrA* and *parC* genes, resistance gene cassettes in the integrons, fluoroquinolone efflux and other genes mediating resistance to different antimicrobials.

Material & Methods

Bacterial strains: Seventeen E. coli isolates obtained from patients from a diarrhoeal outbreak during 2000 in Ahmedabad, Gujarat, were included in this study. The detailed description of the outbreak along with the phenotypic and genetic features of the ETEC strains were published elsewhere². E. coli (J53), Klebsiella pneumonia (NK835), Morgonella morgonii (500914) and Shigella flexneri 3a (IDH6663), harbouring qnr gene alleles A, B, D and S, respectively were used as positive control in the PCR assay. The amplicons of these strains were confirmed by DNA sequencing. Vibrio cholerae O1 (SK-10) and E. coli strain carrying R483::Tn7 were used in the preparation of DNA probes that were the positive controls for the detection of *intI1* and intl2, respectively. E. coli strain DH5a or C600 was used as negative control in all the PCR assays. The ATCC strains E. coli 25922 and Staphylococcus aureus 25923 were used as quality control in the antimicrobial susceptibility assay.

Antimicrobial susceptibility test: The susceptibility of ETEC strains to different antimicrobials was performed by disk diffusion method using commercially available discs (Becton, Dickinson & Co, Sparks, MD, USA). Disc containing ampicillin (A, 10 μ g), chloramphenicol (Ch, 30 μ g), co-trimoxazole (Co, 25 μ g), gentamicin (G, 10 μ g), neomycin (Ne, 30 μ g), tetracycline (T, 30 μ g), streptomycin (S, 10 μ g), nalidixic acid (Na, 30 μ g), ceftazidime (Cf 10 μ g), kanamycin (K, 30 μ g),

ceftriaxone (Ci, 30 μ g), ciprofloxacin (Cx, 5 μ g), norfloxacin (Nx, 10 μ g), cefotaxime (Ct, 30 μ g) and ceftazidime (Tz, 30 μ g) were used. Susceptibility patterns were based on the recommendations of the Clinical Laboratory Standards Institute¹⁶.

The minimal inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin, norfloxacin, cefotaxime and ceftazidime were determined by E-test (AB Biodisk, Solna, Sweden) or agar dilution technique¹⁷ using Mueller-Hinton agar (Difco, USA) supplemented with appropriate concentration of antimicrobials (Nacalai Tesque, Kyoto, Japan). All the ETEC strains were also tested for ESBL-production by modified double-disk synergy test, phenotypic confirmatory disk diffusion test and E-test with Ct and Tz strips.

Polymerase chain reaction (PCR) assay: DNA templates were prepared from 200 μ l of overnight culture of the test strains in Luria Bretani broth (LB, Difco, USA) by centrifugation using mircofuge tubes and resuspending the bacterial pellet to the initial volume with distilled water. The crude template DNA was prepared by boiling for 10 min and directly used in the PCR assay. The reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA). The target genes, primer sequences, PCR conditions and amplified product sizes are given in Table I. The PCR products were analyzed after electrophoresis and staining with ethidium bromide.

Nucleotide sequencing: The PCR products were purified either directly or using gel extraction kits (Qiagen, Hilden, Germany). Sequencing of both the DNA strands was performed with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturers' instructions. The DNA fragments were sequenced and analyzed using an automated sequencer (ABI PRISM 310 DNA, Applied Biosystems). The nucleotide and deduced protein sequences were analyzed with DNASIS (Hitachi, Yokohama, Japan) and DNASTAR (DNA Star Inc. Madison, USA) software. Nucleotide sequences were analyzed by searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service (*www.ncbi.nlm.nih.gov/blast*).

Fluoroquinolone accumulation assay: E. coli cells were grown to mid-log phase in LB ($OD_{600} = 0.4$), harvested, and suspended in 0.2 M morpholinepropanesulphonic acid-Tris buffer (*p*H 7.0) to an optical density at 600 nm of 20 per ml. Cells were energized with 0.2 per cent glucose for 20 min. Fluoroquinolones were

added at a concentration of 10 μ g/ml. Aliquots of this mixture was harvested and suspended in 1 ml of 100 mM glycine-HCl (*p*H 3.0) and shaken for 1 h at room temperature. The amount of released fluoroquinolone was determined spectroflurometrically with excitation at 277 nm and emission at 448 nm. Experiments were done in triplicate and included repeated measurements after carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the assay mixture as an inhibitor of the proton-motive force at a final concentration of 100 μ M.

DNA hybridization analysis: Locations of intIl and *intI2* were identified by colony hybridization assay using Hybond⁺ nylon membranes (Amersham Biosciences Corp, Bucks, UK). DNA probes for *intl1*, intI2 and aac(6')-1b-cr genes were prepared by PCR using primers described in Table I with V. cholerae (SK10), R483:: Tn7 plasmid and ETEC strain AV195 as templates, respectively. The probe DNA (integron) was labelled by digoxigenin (DIG) labelling and detection kit (Roche Diagnostic GmbH, Manheim, Germany) and aac(6')-1b-cr probe was labelled with alkaline phosphatase (GE Healthcare, Buckinghamshire. UK). The identities of the amplified products were confirmed by PCR direct sequencing. Plasmid and genomic DNA of the integron positive isolates were extracted following standard procedures. Genomic DNA was digested with EcoRI and EcoRV enzymes (Takara Suzo Co Ltd, Otsu, Japan). The plasmid and the digested genomic DNA were subjected to 0.7 per cent agarose gel electrophoresis and then blotted on a nylon membrane. Southern hybridization was performed to determine the location of *intI1*, *intI2* and *aac(6')-1b*cr using the respective probes, under high-stringency conditions.

Nucleotide sequence accession numbers: The nucleotide sequence data reported here have been assigned the following GenBank accession numbers: DQ116793 to DQ116794 for *gyrA*, DQ116795 to DQ116798 for *parC* AB188258 to AB188268; AB188271 to AB188272 for resistance gene cassettes, and EF501991 to EF501992 for *acc(6')-Ib-cr*.

Results

The antimicrobial susceptibility results showed that the ETEC strains were resistant to ampicillin (100%), cephalothin, tetracycline, co-trimoxazole, nalidixic acid, norfloxacin, ciprofloxacin (94% each), chloramphenicol (59%), streptomycin, kanamycin (52% each). In addition, most of the ETEC were

Primer (direction) Locus Primer sequence (5'-3') Amplicen (b) Reference (b) Reference (b) PCR condition (b) GA (-) GA (-) gyr.4 ACGIACLAGGCAAIGACIGG (ACAI)AGACITCGCGTATAGAAC 190 18 55 60 CA (-) purC CITATGCGAAIGACITGGGAAIA 265 18 50 30 Int (-) int (-) crtatacodaGACITGGGAAIGACITG 200 This study 50 30 Int (-) int (-) adaCCTATTACAGCACACITG 420 This study 50 30 Int (-) adaCCTATTGCGCAATCAGCGGAACC AAATCITTAACCCGCAAACC Variable 20 55 60 Int (-) Gene cassette GGCATACAAGCAGCATCACCGTG Variable 20 50 30 Int (-) Gene cassette GATCTCATCGCCATTTCCCGAG 73 21 61 30 Int (-) adaCCCATCACCCGTGG Variable 20 50 30 Int (-) Gene cassette GATCTGCACATCCCGTG 74 22 50 30 Int (-) </th <th colspan="8">Table I. List of PCR primer pairs used in this study</th>	Table I. List of PCR primer pairs used in this study							
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$\begin{array}{cccccccccccccccccccccccccccccccccccc$	TetC (-)	tetC	CTACAATCCATGCCAACCC	881			30	
TetD (-) tetD CAGGACACCCTGTAGTTTTC tetD TTAATGGCAACAGCCAGC to tetD TTAATGGCAACAGCCAGC to tetE TTAATGCCATCCCATCCATCCAC to tetE TTAATGCCCATCCATCCAC to tetE TTAATGCCCAACCCAG to tetE to tetE AATCACTGCCATCCAGTTCAC to tetE to tetE TTAATGCCCCACACCCAG to tetE to tetE COCCAGATAAATCACCACACAGC to tetE to tetE TTAATGCCCACACACAC to tetE to	TetD (+)		TGGGCAGATGGTCAGATAAG					
Tete (-) Tete (-)teteTTAATGGCAACAGCCAGC TCCATACCCATCCATTCCAC 853 22 50 30 β -Lac (-) bla_{CTXM-3} AATCACTGCGTCAGTTCAC TTTATCCCCCACAACCCAG 701 22 50 30 β -Lac (-) bla_{CTXM-3} TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG 768 22 50 30 β -Lac (-) $CGCCAGATAAATCACCACAATG$ 768 22 50 30 β -Lac (-) $CGCCAGATAAATCACCACAATG$ 823 22 50 30 TetY (+) $tetY$ $TCCCAAGCGCACACACAC$ 823 22 50 30 Aph (+) $aphA1-1a$ $GGCCAGCTCATTGTGTCGCAGGCGCGGGGCCGCGTCATGGAGAGGAGTTTATCGCGACCTGAAAGCCGGC670216530AdB (+)aadBGGCCGCGCGTCATGGAGGGGAGGAGTTTATCGCGACCTGAAAGCGGC329216530StrA (-)strAATCAACTGGCAGGAGGAGAACAGGGATCGGCAAAGGTTAGGGCAAGG580216530QnrA (+)qnrAATCACTGCGCAGGAGGAGGAACAGGGATCGGCAAAGGTAGGGCAAG51623, 245345QnrS (-)qnrBACGATCGTGAAAGCCAGAAAGGTACGATCGTGAAAGCCAGAAAGG41723, 245345QnrS (-)qnrSACGACGCAAAAACAAAGTTAGGCAAAGCACCCTGTAGGC41723, 245345QnrS (-)qnrSACGACGCAAAAACAAAGTTAGGCAAAGCACCCTGTAGGC41723, 245345QnrS (-)qnrSACGACGCAAAAACAAAGTTAGGCTAAATTGGCACCCTGTAGGC$	TetD (-)	tetD	CAGCACACCCTGTAGTTTTC	827	22	50	30	
TetE (·) tetE TCCATACCCATCCATTCCAC 853 22 50 30 β -Lac (+) $bla_{CTX:M3}$ AATCACTGCGTCAGTTCAC 701 22 50 30 β -Lac (-) bla_{SHV} TCGCCTGTGTATTATCTCCC 768 22 50 30 β -Lac (-) CGCAGATAAATCACCACAATG 768 22 50 30 TetY (+) tetY ACCGCACTCATTGTTGTC 768 22 50 30 TetY (-) $aphAl-la$ ACCGCACTCATTGTTGTC 768 22 50 30 AACGTCTTGCTCGAGGCCGCG 670 21 65 30 AACGTCTTGCTCGAGGCCGCG 670 21 65 30 AddB (-) $addB$ GGCCGCGTCATGGAGAGACACAC AadB (-) $addB$ GGCCGCGCTCATGGAGGAGGAGTT 74TCTCCCC 670 21 65 30 StrA (+) $strA$ CCAATCGCAGAAGAGGGCAGG 580 21 65 30 StrA (+) $strA$ CCAATCGCAGGAGGAGAGAGG 580 21 65 30 QnrA (-) $qnrA$ ATTCACTGGCAGGAGGAGCAGG 580 21 65 30 QnrA (-) $qnrA$ ATTCACCGCAGGATTGG 516 23, 24 53 45 QnrB (-) $qnrB$ ACGATCCTGGTAGGGCAGGA QnrS (-) $qnrS$ ACGACATCGCTGGTAGTGCC 469 23, 24 53 45 QnrS (-) $qnrS$ ACGACATCGCGAAAAGCTAGGC 417 23, 24 53 45 Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTTAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTAAGCAAGTAAGCAAGTTAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTAAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTTAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTTAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTAAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTAAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAAACAAAGTAAGG Faa(-) $ac(6')$ - <i>Ib-cr</i> GCAACGCAAAACCATGTACA $ccATGTACA CATGTACA CATGTACA CATGTACA ccATGTACA CATGTACA CATGTACA CATGTACA CATGTACA ccATGTACA CATGTACA CATGTACA CATGTACA$	TetE (+)	_	TTAATGGCAACAGCCAGC					
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	TetE (-)	tetE	TCCATACCCATCCATTCCAC	853	22	50	30	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	β-Lac (+)		AATCACTGCGTCAGTTCAC					
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	β-Lac (-)	bla _{CTX-M-3}	TTTATCCCCCACAACCCAG	701	22	50	30	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	β -Lac (+)	blagury	TCGCCTGTGTATTATCTCCC				• •	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	β-Lac (-)	orashy	CGCAGATAAATCACCACAATG	768	22	50	30	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	TetY(+)		ACCGCACTCATTGTTGTC					
Aph (+) Aph (-) $aphA1-1a$ AACGTCTTGCTCGAGGCCGCG GGCAAGATCCTGGTATCGGTCTGC670216530AadB (+) AadB (+) $aadB$ GGGCGCGTCATGGAGGAGGTT TATCGCGACCTGAAAGCGGC329216530StrA (+) StrA (-) $strA$ CCAATCGCAGATAGAAGGCAAG ATCAACTGGCAGGAGGAGCAAGG580216530QnrA (+) QnrA (-) $qnrA$ ATTTCTCACGCCAGGAGGAGAACAGG GATCGGCAAAGGTTAGGTCA51623, 245345QnrB (+) QnrB (-) $qnrB$ GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC6923, 245345QnrS (+) QnrS (-) $qnrS$ ACGACGTCACTGTAACTGCAA TAAATTGGCACCCTGTAGGC41723, 245345Faa(-) $ac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA561This study5045	TetY (-)	tetY	TTCCAAGCAGCAACACAC	823	22	50	30	
Aph (-) $aphAI-Ia$ GGCAAGATCCTGGTATCGGTCTGC 670 21 65 30 AadB (+) $aadB$ GGGCGCGTCATGGAGAGAGTT 329 21 65 30 AadB (-) $aadB$ TATCGCGACCTGAAAGCGGC 329 21 65 30 StrA (+) $strA$ CCAATCGCAGATAGAAGCGAC 580 21 65 30 QnrA (-) $qnrA$ ATTCTCACGCAGAAGGAACAGG 580 21 65 30 QnrA (-) $qnrA$ ATTCTCACGCCAGGATTG 516 $23, 24$ 53 45 QnrB (+) $qnrB$ GATCGTGAAAGCCAGAAAGG 469 $23, 24$ 53 45 QnrS (+) $qnrS$ ACGACATTCGTCAACTGCAA 417 $23, 24$ 53 45 Faa(+) $aac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG 561 This study 50 45	Aph (+)	1 4 1 1	AACGTCTTGCTCGAGGCCGCG	(70)	21	<i>(</i> -	20	
AadB (+) AadB (-) $aadB$ GGGCGCGTCATGGAGGAGTT TATCGCGACCTGAAAGCGGC 329 21 65 30 StrA (+) StrA (-) $strA$ CCAATCGCAGATAGAAGGCAAG ATCAACTGGCAGGAGGAACAGG 580 21 65 30 QnrA (+) QnrA (-) $qnrA$ ATTTCTCACGCCAGGATTGG GATCGGCAAAGGTTAGGTCA 516 $23, 24$ 53 45 QnrB (+) QnrB (-) $qnrB$ GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC 469 $23, 24$ 53 45 QnrS (+) QnrS (-) $qnrS$ ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC 417 $23, 24$ 53 45 Faa(+) Faa(-) $aac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA 561 This study 50 45	Aph (-)	aphA1-1a	GGCAAGATCCTGGTATCGGTCTGC	670	21	65	30	
AadB (-)addbTATCGCGACCTGAAAGCGGC 329 21 63 30 StrA (+) $strA$ CCAATCGCAGATAGAAGCGAC 529 21 65 30 StrA (-) $strA$ ATCAACTGGCAGGAGGAAGGGAACAGG 580 21 65 30 QnrA (+) $qnrA$ ATTTCTCACGCCAGGATTG 516 $23, 24$ 53 45 QnrA (-) $qnrA$ GATCGTGAAAGCCAGAAAGG 469 $23, 24$ 53 45 QnrB (+) $qnrB$ GATCGTGAAAGCCAGAAAGG 469 $23, 24$ 53 45 QnrS (+) $qnrS$ ACGACATTCGTCAACTGCAA 417 $23, 24$ 53 45 QnrS (-) $qnrS$ ACGACGCAAAAACAAAGTTAGG 417 $23, 24$ 53 45 Faa(+) $aac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG 561 This study 50 45	AadB (+)	a a dD	GGGCGCGTCATGGAGGAGTT	220	21	65	30	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	AadB (-)	ааад	TATCGCGACCTGAAAGCGGC	529	21	05	30	
StrA (-) $StrA$ ATCAACTGGCAGGAGGAGGAGCAGGG 560 21 63 30 QnrA (+) $qnrA$ ATTTCTCACGCCAGGATTTG 516 $23, 24$ 53 45 QnrA (-) $qnrA$ GATCGGCAAAGGTTAGGTCA 516 $23, 24$ 53 45 QnrB (+) $qnrB$ GATCGTGAAAGCCAGAAAGG 469 $23, 24$ 53 45 QnrS (+) $qnrS$ ACGACATTCGTCAACTGCAA 417 $23, 24$ 53 45 QnrS (-) $qnrS$ ACGACGCAAAAACAAGGTAGGC 417 $23, 24$ 53 45 Faa(+) $aac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA 561 This study 50 45	StrA (+)	stu A	CCAATCGCAGATAGAAGGCAAG	580	21	65	20	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	StrA(-)	SUA	ATCAACTGGCAGGAGGAACAGG	380	21	05	30	
QnrA (-)QnrAGATCGGCAAAGGTTAGGTCA51023, 245345QnrB (+) $qnrB$ GATCGTGAAAGCCAGAAAGG46923, 245345QnrB (-) $qnrB$ ACGATGCCTGGTAGTTGTCC46923, 245345QnrS (+) $qnrS$ ACGACATTCGTCAACTGCAA41723, 245345QnrS (-) $qnrS$ TAAATTGGCACCCTGTAGGC41723, 245345Faa(+) $aac(6')$ -Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA561This study5045	QnrA (+)	anr 1	ATTTCTCACGCCAGGATTTG	516	23 24	53	15	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	QnrA (-)	<i>qmA</i>	GATCGGCAAAGGTTAGGTCA	510	2J, 2 4	55	43	
QnrB (-)QnrDACGATGCCTGGTAGTTGTCC10725, 2155107QnrS (+)QnrS (-)ACGACATTCGTCAACTGCAA41723, 245345Faa(+)GCAACGCAAAAACAAAGTTAGGGCAACGCAAAAACAAAGTTAGG561This study5045	QnrB (+)	anrB	GATCGTGAAAGCCAGAAAGG	469	23, 24	53	45	
QnrS (+) QnrS (-)qnrSACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC41723, 245345Faa(+) Faa(-)aac(6')-Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA561This study5045	QnrB (-)	quib	ACGATGCCTGGTAGTTGTCC	109				
QnrS (-)TAAATTGGCACCCTGTAGGCTA'25, 215515Faa(+)aac(6')-Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA561This study5045	QnrS (+)	anrS	ACGACATTCGTCAACTGCAA	417	23, 24	53	45	
Faa(+) Faa(-)aac(6')-Ib-crGCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA561This study5045	QnrS (-)	√ ~	TAAATTGGCACCCTGTAGGC	•••				
Faa(-) GTGTTTGAACCATGTACA	Faa(+)	aac(6')-Ib-cr	GCAACGCAAAAACAAAGTTAGG	561	This study	50	45	
	Faa(-)	. /	GIGITIGAACCAIGIACA		2			

[¶]+, coding strand; -, noncoding strand; [†]annealing temperature in °C; [‡]extension time in sec

also resistance to other antibiotics (Table II). The multidrug resistant ETEC were not associated with a particular serogroup but overall the serogroup O1 was comparatively high (53%). The MICs for nalidixic acid resistance was high (2000 μ g/ml) and for fluoroquinolones, the MIC ranged between 250 and 1000 μ g/ml. In six ETEC strains, the MIC for Ct and Tz were ranged from >64-340 and >84-168 μ g/ml, respectively (Table III).

DNA sequencing of the 190-bp PCR product covering the QRDR of gyrA demonstrated presence of mutations at codons 83 in all the quinolone and fluoroquinolone resistant strains compared to the sensitive strain AV195 (Table IV). Mutation at codon 83 was a C \rightarrow T transversion in the codon TCG that resulted substitution of leucine for serine. Except for one strain (E17), the second mutation was noted in all the strains at position 87 (G \rightarrow A transversion of codon GAC), which resulted in an asparagine substitution for an aspartate. A 265-bp PCR amplicon of parC was also analyzed in this study. Majority of the quinolone resistant strains had a mutation at codon 80 (G \rightarrow T translation of codon AGC), resulting substitution of isoleucine for serine. A second mutation at position 84 $(E \rightarrow G)$ in the *parC* was detected in E13 and AV185, in which glutamic acid was replaced by glycine. In six ETEC strains, mutation was detected at position 108 $(A \rightarrow V)$), where alanine was substituted by value. In three strains (E2, E14, and AV185) the mutation at position 108 was absent. Strain AV185 had a third mutation at position 90 (A \rightarrow V), where alanine was replaced by valine. Interestingly, the strain AV195 that is susceptible for nalidixic acid (MIC 4µg/ml) exhibited low-levels of resistance to fluoroquinolones. Since none of the fluoroquinolone resistance mechanisms were identified in this strain, we assume that it may harbour a novel PMQR and/or other hitherto unknown functional gene(s).

None of the ETEC strains were positive in the Qnr-PCR assay, indicating the absence of all the tested *qnr* alleles. In addition to the mutations in QRDR, efflux pumps play an important role in intrinsic resistance of *E. coli* to fluoroquinolones. The fluoroquinolone accumulation kinetics before and after the addition of CCCP was almost similar in most of the resistant strains (Table IV) indicating the involvement of other resistance mechanism(s). Most of the ETEC strains (64.7%) harboured the newly described aac(6')-*Ib*-cr gene, which encodes the fluoroquinolone-modifying enzyme aminoglycoside acetyltransferase (Table II). In majority of ETEC strains the acc(6')-*Ib*-cr gene was located in large plasmids of varying sizes and seven ETEC strains had this gene in the chromosome (Table II). All the six ETEC strains, which were resistant to Ct and Tz harboured $bla_{CTX-M-3}$ (Table III).

In the colony hybridization assay, class 1 and class 2 integrons were detected in 35.3 and 17.6 per cent of the ETEC strains, respectively. Four strains (23.5%) had both the classes of integrons (Table II). ETEC strains carrying the integrons were further screened for the presence of contiguous resistance gene cassettes, using specific primers inF/inB and intl2/Hep51, respectively. Of the 10- *int11* probe positive strains, six carried two cassettes and the resistance genes detected in these strains were *dfrA17* and *aadA5*, which confer resistance to trimethoprim and spectinomycin/streptomycin using dihydrofolatereductase and adenylytransferase, respectively.

In addition to these resistance gene cassettes, two strains (E9 and E17) gave an additional amplicon with a size of 1009 bp, which was identified as *aadA1*, conferring resistance for aminoglycosides. E3 and E6 strains carried 1009 and about 800 bp with complete and incomplete *aadA1* gene cassette, respectively. In one *int11* probe positive strain (E15), the CS specific primers did not gave amplicon. In seven ETEC strains, *int12* was detected, of which four were also positive for *int11*. All the *int12* positive strains carried three cassettes (2449 bp) as those found in Tn7, namely *dfrA1, sat1*, and *aadA1* (Table II). The *sat* gene encodes for streptothricin acetyltransferase.

The plasmid patterns and the location of the *intI1* gene in the plasmids were not uniform (Table II). The restriction enzymes, *Eco*RI and *Eco*RV were used for digestion of chromosomal DNA, since the *intI1* did not have the corresponding restriction sites for these enzymes. Southern hybridization results showed that class 1 integron was detected both in the chromosome and large plasmid in most of the strains (Table II), however, class 2 integron was detected only in the chromosome. In E15, *intI1* was detected in the large plasmid, however, this strain did not carry any resistance gene cassette.

The alleles of genes such as *tet*, *catA1*, *strA*, *bla*_{*TEM*}, and *aphA1-Ia* encoding for resistance for tetracycline, chloramphenicol, streptomycin, ampicillin, and kanamycin, were detected in 94.1, 70.6, 58.8, 35.2, 29.4 per cent of the ETEC strains, respectively. An analysis on the presence of different resistance gene

Strain	Resistance profile	Resistar	ice gene cassette	Other resistance gene	Location of intII	Location of
(Serogroup)		intII	int12	1		aac (6')-1b-cr
E2 (0146)	ACeCxTCoNxNaNe	dfrA17, aadA5,	1	tetB	Chromosome, large plasmid	
E3 (01)	ACeCfCiCxChTCoSNxNaNe	aadAI	dfrAI,satI, aadAI	catAl, tetB, strA	Chromosome Plasmid (8.6 kb)	
E4 (01)	ACeAmKCxChTCoSNxNaNe	dfrA17, aadA5		catAl, tetAB, strA, aphA1-la, aac(6')-lb-cr	Large plasmid	
E6 (08)	ACeCxChTCoSNxNa	aadAI		bla_{TEM} catA1, tetB, strA	Chromosome, large plasmid	
E7 (0146)	ACeKCiCxTCoSNxNa	dfrA17, aadA5	dfrAI, sat1, aadAI	bla_{TEM} tetB, strA, $aac(6')$ -Ib-cr	Chromosome, large plasmid	
E8 (ONT)	ACeGCfKCiCxChTCoNxNaCtTz	dfrA17, aadA5		bla_{TEM} tetB, catA1, aac(6')-Ib-cr, $bla_{CTE-M-3}$	Chromosome, large plasmid	Chromosome, large plasmid (~91 kb)
E9 (0146)	ACeGCxChTCoSNxNa	dfrA17, aadA5, aadA1	dfrAI, satI, aadAI	bla _{TEM} catAl, tetB, strA, aac(6')-Ib-cr	Chromosome, large plasmid	Chromosome, large plasmid (~73kb)
E10 (O1)	ACeCfKCiCxChTCoNxNa	dfrA17, aadA5		catAl, tetB, aac(6')-Ib-cr	Chromosome	Chromosome, large plasmid (~88kb)
E13 (01)	AceGCfKCiCxChTCoSNxNaNe CtTz	ı	dfrAI, satI, aadAI	caA1, tetAB, strA aphA1-Ia, aac(6')-Ib-cr, bla _{CTK-M-3}		Chromosome, large plasmid (~90kb)
E14 (O1)	AceGCfKCiCxChTCoSNxNaNe CtTz	ı	dfrAI, sat1, aadAI	catAI, tetAB, strA,aphAI-Ia, aac(6')-Ib-cr, bla _{CTKM-3}		large plasmid (~101kb)
E15 (01)	ACeCxTNxNa	ı		tetBE, aac(6')-lb-cr	large plasmid	
E16 (01)	ACeCfChTCoNxNaCx	dfrA17, aadA5		catAI, $tetB$	Chromosome	
E17 (0146)	ACeGKCxChTCoSNxNa	dfrA17, aadA5, aadA1	dfrAI, satI, aadAI	bla_{TEM} catA1, tetB, strA	Chromosome, large plasmid	
AV185 (01)	ACoCfCxKTNxSNaCtTz	ı	dfrAI, satI, aadAI	catAl, tetAB, strA, aphAl-la, aac(6')-lb-cr, bla _{CTX-M-3}		large plasmid (~90kb)
AV189 (025)	ACeGCfKCiCxTNxNaCtTz	ı	ı	tetA, $aac(6')$ -Ib-cr, $bla_{crX:M:3}$		large plasmid (~83kb)
AV193 (ONT)	ACeCoCxNxNaGKTCfTz	I		bla _{TEM} , catAI, strA, tetABE, aphAI-Ia, aac(6')-Ib-cr, bla _{CTXM-3}		large plasmid (~83kb)
AV195 (01)	ACeCo	ı	ı			
ONT, untypał Am, amikacin;	ole; A, ampicillin; Ch, chloramphen Cf, ceftazidime; K, kanamycin; Ci, c	uicol; Co, co-trimc ceftriaxone; Cx, cip	xazole; G, gentamicir rofloxacin; Nx, norflox	1; Ne, neomycin; T, tetracycline; S, stre acin; Ct, cefotaxime; Tz, ceftazidime	eptomycin; Na, nalid	ixic acid; Ce, cephaloth

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combinations showed that ETEC harbouring bla_{TEM} gene was detected with *catA1* gene in 5 out of 6 strains and the strains harbouring *aphA1-Ia* were also positive for *tetAB* or *tetABE*. In one streptomycin resistant strain (E2) *strA* gene was negative but carried *aadA5*, which was associated with the class 1 integron structure. The four strains, E2, E8, E10 and E16 were susceptible to streptomycin and did not harbour *aadA1* or *strA*. In 6 gentamicin resistant strains, *aadB*, which confer gentamicin resistance, was not identified.

Table III. MICs of fluroquinolone and ESBLs of ETEC strains								
Strain		MIC (µg/ml)						
	Na	Cx	Nx	Ct	Tz			
E2	2000	250	250	*	*			
E3	2000	250	250	*	*			
E4	2000	500	500	*	*			
E7	2000	1000	1000	*	*			
E8	ND	ND	ND	>64	>84			
E9	2000	1000	500	*	*			
E13	2000	500	500	>84	>128			
E14	2000	1000	500	>102	>168			
E15	2000	250	250	*	*			
E16	2000	250	200	*	*			
E17	2000	250	250	*	*			
AV185	2000	1000	500	>250	>112			
AV189	ND	ND	ND	>340	>128			
AV193	ND	ND	ND	>170	>128			
AV195	4	2	2	1.3	<2			

Ct, cefotaxime; Tz, ceftazidime; Nx, norfloxacin; ND, not done; *susceptible to cefotaxime and ceftazidime

Among *tet* gene alleles, *tetB* was detected in 52.9 per cent of the ETEC strains. Combination of *tetAB* was detected among four strains, and *tetBE*, *tetABE*, *tetA* was detected in one strain each (Table II). The other tested *tet* gene types such as *tetC*, *tetD* and *tetY* were not detected in any of the ETEC strains (data not shown). When tested for the MIC of tetracycline by agar dilution technique with strains harbouring different *tet* gene classes, *tetA* or *tetB* alone had the MIC of 100 µg/ml. ETEC strains with combination of *tetBE* and *tetABE* had the MIC of 150 µg/ml (data not shown).

Discussion

All the ETEC strains isolated from the patients with diarrhoea during the outbreak were resistant to several antibiotics. The differences found in their *XbaI* PFGE patterns indicate that most of the ETEC strains in this study are distinct clones². The unusual multidrug resistance profiles of the ETEC strains prompted us to undertake an in depth study on mechanisms of antimicrobial resistance using the molecular tools.

Fluoroquinolones have been used for the treatment of variety of infectious diseases. In the present study, most of the ETEC were highly resistant to fluoroquinolones. Prior to 1990, resistance of *E. coli* to fluoroquinolones was rare²⁵. As reported with other pathogens, use of fluoroquinolones in India may have progressively favoured emergence of MDR-ETEC strains⁴⁻⁶. Majority of the ETEC investigated in the

Table IV. Amino acid substitutions in the QRDRs and fluroquinolone efflux of ETEC strains								
Strain	Amino acid substitution		Accumulation of fluroquinolones [µg/mg (dry weight) of cells]					
			C	Сх		x		
	GyrA	ParC	Before addition of	After addition of	Before addition of	After addition of		
			CCCP	CCCP	CCCP	CCCP		
E2	S83→L, D87→N	S80→I, A108	0.064 ± 0.0011	0.089 ± 0.0028	0.161 ± 0.007	0.502 ± 0.0032		
E3	S83→L, D87→N	S80 \rightarrow I, A108 \rightarrow V	0.067 ± 0.003	0.089 ± 0.0025	0.166 ± 0.003	0.461 ± 0.0021		
E4	S83→L, D87→N	S80 \rightarrow I, A108 \rightarrow V	ND	ND	ND	ND		
E7	S83→L, D87→N	S80 \rightarrow I, A108 \rightarrow V	0.014 ± 0.0013	0.020 ± 0.0019	0.043 ± 0.0010	0.047 ± 0.0022		
E9	S83 \rightarrow L, D87 \rightarrow N	S80 \rightarrow I, A108 \rightarrow V	ND	ND	ND	ND		
E13	S83→L, D87→N	S80→I, E84→G	0.009 ± 0.0012	0.020 ± 0.0020	0.024 ± 0.0011	0.044 ± 0.0014		
E14	S83→L, D87→N	S80→I, A108	0.012 ± 0.0015	0.014 ± 0.0035	0.036 ± 0.0017	0.040 ± 0.0033		
E15	S83→L, D87→N	S80 \rightarrow I, A108 \rightarrow V	ND	ND	ND	ND		
E16	S83→L, D87→N	S80 \rightarrow I, A108 \rightarrow V	ND	ND	ND	ND		
E17	S83→L, D87	S80, A108	0.013 ± 0.0011	0.022 ± 0.0021	0.046 ± 0.0014	0.047 ± 0.0024		
AV185	S83→L, D87→N	S80→I, E84→G, A90→V, A108	0.031 ± 0.005	0.090 ± 0.0011	0.133 ± 0.002	0.531 ± 0.0010		
AV195	S83, D87	S80, A108	0.088 ± 0.007	0.096 ± 0.009	0.564 ± 0.004	0.590 ± 0.006		
ND, not done; Cx, ciprofloxacin; Nx, norfloxcin, CCCP, carbonyl cyanide <i>m</i> -chlorophenylhydrazone. Values are mean, n=3								

present study were resistant to several other antibiotics as well. It has been demonstrated that fluoroquinolone resistance was related with resistance to at least one non-fluoroquinolone antibiotic²⁶. In this study, the fluoroquinolone resistant ETEC strains had mutations in gyrA at positions 83 and 87. The gyrA mutations that frequently affect residue serine at 83 and aspartate at 87 are common among fluoroquinolone resistant E. $coli^{27}$. In addition to the mutations in gyrA, mutations were detected in the *parC*. For the expression of high-level resistance, acquisition of a second gyrA mutation and a *parC* mutation seems important. A similar resistance mechanism has been observed in E. *coli* strains²⁸. With the sequenced regions of QRDR in this study, association could not be made with the increased MIC of quinolone/fluoroquinolones and amino acid substitutions in topoisomerases. Although the quinolone resistance is due to mutations in gyrA and *parC*, studies indicate that resistance can also be transferred on plasmids carrying the *qnr* gene alleles7,8.

Although *E. coli* has shown to have intrinsic protondependent multidrug-resistant efflux pump systems, the specific activity of any one pump in its natural state and level has not been established²⁹. CCCP is a well-known inhibitor for fluoroquinolone efflux pump in many bacteria. After the addition of this proton motive force uncoupler, the expected accumulation did not occur in most of the fluoroquinolone resistant ETEC. In view of the large number of proven or putative efflux pumps³⁰, involvement of more than one mechanism seems possible. We assume that additional non-efflux resistance mechanism(s) may also contribute to the high level fluoroquinolone resistance.

Class 1 and class 2 integrons are widely prevalent in most of the clinical strains of Gram-negative bacteria. Despite the differences in the integrase proteins, the two classes of integrons can include identical gene cassettes³¹. Our finding has shown that the *dfr* and *aad* gene alleles encoding dihydrofolate reductases and adenyltransferase, respectively were present in both the classes of integrons among ETEC. In this study, *dfr* cassettes (*dfrA1* and *dfrA17*) that confer resistance to trimethoprim were detected in majority of the ETEC strains. In 50 per cent of the *intl1* positive ETEC, *dfr17* was detected along with *aadA5* cassette. The *dfr* and *aadA* cassettes were shown to be common among members of the family *Enterobacteriaceae*³². The *dfr* was detected behind the 5'-conserved segment, which is closest to the promoter, thereby providing high-level and conditional resistance. It is likely that selection for cassettes carrying *dfr* genes has occurred among ETEC as trimethoprim in combination with sulphamethoxazole (co-trimoxazole) is used frequently in the treatment of diarrhoea and other infections. Presence of *aadA5* did not have any influence towards resistance of streptomycin, as the *aad* cassette confers resistance only to low level of streptomycin³³. One ETEC strain (E15), which was *intI1* probe positive, did not amplify any resistance gene cassette regions, possibly due to the variable nature of a 3'-conserved segment.

In *intl2* positive ETEC strains, we have detected *dfrA1*, *sat1* and *aadA1* gene cassettes conferring resistance to trimethoprim, streptothricin, and streptomycin/spectinomycin, respectively. Existence of *sat1* has not been reported in ETEC. Class 2 integron carrying *dfrA1*, *sat1* and *aadA1* was also reported among members of the family *Enterobacteriaceae* due to the defective integrase gene in class 2 integron²⁰.

Within the integrons, the genes responsible for resistance to beta-lactams, tetracycline, and chloramphenicol were not mapped in this study. The *tetB* gene that encodes an efflux protein, which confers resistance to tetracycline, was predominant in majority of our ETEC strains. As reported in other findings, combination of tet genes was detected in six ETEC strains²². Except for one strain (AV189), tetA was always detected with tetB in this study. Interestingly, ETEC harbouring combination of *tetA*, tetB or tetA, tetB, and tetE expressed high MIC for tetracycline than tetB alone or tetB and tetE. None of the six gentamicin resistant strains carried the *aadB* gene. Possibly, the other variant genes encoding aminoglycoside adenyltransferase may play a role in resistance towards gentamicin. We have tested the ETEC strains for ESBL genes such as *bla_{SHV}* and *bla_{CTX}*. $_{M-3}$ genes for bla_{TEM} , which were widely distributed in ETEC²². Due to co-selection, bla_{TEM} was found in association with the catA1 in most of the ETEC strains²². Similarly, aphA1-Ia was detected along with different *tet* alleles other than *tetB*. We have detected the aminoglycosides (neomycin and kanamycin) resistance gene (aphA1-Ia) in five strains (29.4%), even though 59 per cent of the ETEC were resistant to kanamycin. This may be due to cross-resistance caused by aminoglycoside resistance genes. In this study, 64.7 per cent of the ETEC strains harboured aac(6')-Ib-cr; of which, 73 per cent of the strains concomitantly had ESBL genes. The synergistic effect of these genes on increased resistance toward fluoroquinolones has been established in many members of *Enterobacteriaceae*³⁴.

Even though the antimicrobial therapy is generally supportive for the treatment of ETEC mediated diarrhoea, its rational use is difficult to implement in diarrhoea endemic countries especially during outbreaks. As evidenced from this study, innate gene mutations and acquisition of multidrug resistance genes through mobile genetic elements might have contributed to the emergence of MDR-ETEC. This study reinforces the necessity of utilizing molecular techniques in the epidemiological studies to understand the nature of resistance responsible for antimicrobial resistance in different species of pathogenic bacteria.

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Reprint requests: Dr T. Ramamurthy, National Institute of Cholera & Enteric Diseases, P-33, C.I.T. Road, Scheme XM, Beliaghata, Kolkata 700 010, India e-mail: tramu@vsnl.net