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

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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 (*intI1*) and class 2 (*intI2*) 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%), $bla_{TEM\text{-}1}$ (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 travelers[1]. The first outbreak of ETEC mediated diarrhoea in India was reported from Ahmedabad, Gujarat, during 2000 and majority of the strains expressed multidrug resistance[2]. 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 pathogens[1].

Quinolones and fluoroquinolones are the first-line drugs for the treatment of diarrhoea for many years. However, several studies have documented the emergence and spread of fluoroquinolone resistant enteric pathogens in India[3-6]. Quinolone resistance mechanisms have been described and characterized in a variety of bacteria[7]. 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 chromosome[7]. Most of the quinolone 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 Qnr[8]. Qnr determinant confers resistance to nalidixic acid and increases the MICs of fluoroquinolones by 4-8 folds and supplements the resistance with other mechanisms[8]. 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 pathogens[11].

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 genes[12]. 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 type[13]. Class 2 integrons exists in the transposon Tn*7* or its derivatives, and the 3′-CS contains five *tns* genes involved in the movement of the transposon[14]. 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'-CS[15].

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 elsewhere[2]. *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 *intI2*, respectively. *E. coli* strain DH5α 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 µg), chloramphenicol (Ch, 30 µg), co-trimoxazole (Co, 25 µg), gentamicin (G, 10 µg), neomycin (Ne, 30 µg), tetracycline (T, 30 µg), streptomycin (S, 10 µg), nalidixic acid (Na, 30 µg), cephalothin (Ce, 30 µg), amikacin (Am, 30 µg), ceftazidime (Cf 10 µg), kanamycin (K, 30 µg),

ceftriaxone (Ci, 30 µg), ciprofloxacin (Cx, 5 µg), norfloxacin (Nx, 10 µg), cefotaxime (Ct, 30 µg) and ceftazidime (Tz, 30 µg) were used. Susceptibility patterns were based on the recommendations of the Clinical Laboratory Standards Institute[16].

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 technique[17] 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 µl 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 µg/ml. Aliquots of this mixture were 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 µM.

DNA hybridization analysis: Locations of *intI1* and *intI2* were identified by colony hybridization assay using Hybond$^+$ nylon membranes (Amersham Biosciences Corp, Bucks, UK). DNA probes for *intI1*, *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 *Eco*RI and *Eco*RV 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

Table I. List of PCR primer pairs used in this study

Primer (direction)¶	Locus	Primer sequence (5′-3′)	Amplicon (bp)	Reference	PCR condition	
					T†	T‡
GA (+)	*gyrA*	ACGTACTAGGCAATGACTGG	190	18	55	60
GA (-)		AGAAGTCGCCGTCGATAGAAC				
PC (+)	*parC*	TGTATGCGATGTCTGAACTG	265	18	55	60
PC (-)		CTCAATAGCAGCTCGGAATA				
Int1 (+)	*intI1*	GTTCGGTCAAGGTTCTG	920	This study	50	30
Int1 (-)		GCCAACTTTCAGCACATG				
Int2 (+)	*intI2*	ATGTCTAACAGTCCATTTTT	420	This study	50	30
Int2 (-)		AAATCTTTAACCCGCAAAC				
InF(+)	Gene cassette	GGCATACAAGCAGCAAGC	Variable	19	50	30
InB (-)		AAGCAGACTTGACCTGAT				
IntI2 (+)	Gene cassette	ACCTTTTTGTCGCATATCCGTG	Variable	20	55	60
Hep51 (-)		GATGCCATCGCAAGTACGAG				
TEM (+)	bla_{TEM}	CATTTCCGTGTCGCCCTTATTCC	828	21	59	30
TEM (-)		GGCACCTATCTCAGCGATCTGTCTA				
CAT (+)	*catA1*	AAGTTGGCAGCATTCACCCG	573	21	61	30
CAT (-)		TCGTGGTATTCACTCCAGAGCG				
TetA (+)	*tetA*	GGTCTTGCTCGTCTCGCTGG	690	21	62	30
TetA (-)		AACGCCATCCATCCCCGTG				
TetB (+)	*tetB*	CCTTATCATGCCAGTCTTGC	774	22	50	30
TetB (-)		ACTGCCGTTTTTTCGCC				
TetC (+)	*tetC*	ACTTGGAGCCACTATCGAC	881	22	50	30
TetC (-)		CTACAATCCATGCCAACCC				
TetD (+)	*tetD*	TGGGCAGATGGTCAGATAAG	827	22	50	30
TetD (-)		CAGCACACCCTGTAGTTTTC				
TetE (+)	*tetE*	TTAATGGCAACAGCCAGC	853	22	50	30
TetE (-)		TCCATACCCATCCATTCCAC				
β-Lac (+)	$bla_{CTX-M-3}$	AATCACTGCGTCAGTTCAC	701	22	50	30
β-Lac (-)		TTTATCCCCCACAACCCAG				
β-Lac (+)	bla_{SHV}	TCGCCTGTGTATTATCTCCC	768	22	50	30
β-Lac (-)		CGCAGATAAATCACCACAATG				
TetY (+)	*tetY*	ACCGCACTCATTGTTGTC	823	22	50	30
TetY (-)		TTCCAAGCAGCAACACAC				
Aph (+)	*aphA1-1a*	AACGTCTTGCTCGAGGCCGCG	670	21	65	30
Aph (-)		GGCAAGATCCTGGTATCGGTCTGC				
AadB (+)	*aadB*	GGGCGCGTCATGGAGGAGTT	329	21	65	30
AadB (-)		TATCGCGACCTGAAAGCGGC				
StrA (+)	*strA*	CCAATCGCAGATAGAAGGCAAG	580	21	65	30
StrA (-)		ATCAACTGGCAGGAGGAACAGG				
QnrA (+)	*qnrA*	ATTTCTCACGCCAGGATTTG	516	23, 24	53	45
QnrA (-)		GATCGGCAAAGGTTAGGTCA				
QnrB (+)	*qnrB*	GATCGTGAAAGCCAGAAAGG	469	23, 24	53	45
QnrB (-)		ACGATGCCTGGTAGTTGTCC				
QnrS (+)	*qnrS*	ACGACATTCGTCAACTGCAA	417	23, 24	53	45
QnrS (-)		TAAATTGGCACCCTGTAGGC				
Faa(+)	*aac(6')-Ib-cr*	GCAACGCAAAAACAAAGTTAGG	561	This study	50	45
Faa(-)		GTGTTTGAACCATGTACA				

¶+, 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 µg/ml) and for fluoroquinolones, the MIC ranged between 250 and 1000 µg/ml. In six ETEC strains, the MIC for Ct and Tz were ranged from >64-340 and >84-168 µg/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→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→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→T translation of codon AGC), resulting substitution of isoleucine for serine. A second mutation at position 84 (E→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→V)), where alanine was substituted by valine. In three strains (E2, E14, and AV185) the mutation at position 108 was absent. Strain AV185 had a third mutation at position 90 (A→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 $bla_{CTX\text{-}M\text{-}3}$ (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- *intI1* 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 *intI1* probe positive strain (E15), the CS specific primers did not gave amplicon. In seven ETEC strains, *intl2* was detected, of which four were also positive for *intI1*. All the *intI2* positive strains carried three cassettes (2449 bp) as those found in Tn*7*, 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

Table II. Resistance profile, integrons and drug resistance gene cassettes in ETEC strains

Strain (Serogroup)	Resistance profile	Resistance gene cassette		Other resistance gene	Location of *intI1*	Location of *aac (6')-1b-cr*
		intI1	*intI2*			
E2 (O146)	ACeCxTCoNxNaNe	*dfrA17, aadA5,*	-	*tetB*	Chromosome, large plasmid	
E3 (O1)	ACeCfCiCxChTCoSNxNaNe	*aadA1*	*dfrA1,sat1, aadA1*	*catA1, tetB, strA*	Chromosome Plasmid (8.6 kb)	
E4 (O1)	ACeAmKCxChTCoSNxNaNe	*dfrA17, aadA5*	-	*catA1, tetAB, strA, aphA1-Ia, aac(6')-Ib-cr*	Large plasmid	
E6 (O8)	ACeCxChTCoSNxNa	*aadA1*	-	bla_{TEM}, *catA1, tetB, strA*	Chromosome, large plasmid	
E7 (O146)	ACeKCiCxTCoSNxNa	*dfrA17, aadA5*	*dfrA1, sat1, aadA1*	bla_{TEM}, *tetB, strA, aac(6')-Ib-cr*	Chromosome, large plasmid	
E8 (ONT)	ACeGCfKCiCxChTCoNxNaCtTz	*dfrA17, aadA5*	-	bla_{TEM}, *tetB, catA1, aac(6')-Ib-cr*, $bla_{CTX-M-3}$	Chromosome, large plasmid	Chromosome, large plasmid (~91 kb)
E9 (O146)	ACeGCxChTCoSNxNa	*dfrA17, aadA5, aadA1*	*dfrA1, sat1, aadA1*	bla_{TEM}, *catA1, tetB, strA, aac(6')-Ib-cr*	Chromosome, large plasmid	Chromosome, large plasmid (~73kb)
E10 (O1)	ACeCfKCiCxChTCoNxNa	*dfrA17, aadA5*	-	*catA1, tetB, aac(6')-Ib-cr*	Chromosome	Chromosome, large plasmid (~88kb)
E13 (O1)	AceGCfKCiCxChTCoSNxNaNe CtTz	-	*dfrA1, sat1, aadA1*	*caA1, tetAB, strA aphA1-Ia, aac(6')-Ib-cr*, $bla_{CTX-M-3}$		Chromosome, large plasmid (~90kb)
E14 (O1)	AceGCfKCiCxChTCoSNxNaNe CtTz	-	*dfrA1, sat1, aadA1*	*catA1, tetAB, strA,aphA1-Ia, aac(6')-Ib-cr*, $bla_{CTX-M-3}$		large plasmid (~101kb)
E15 (O1)	ACeCxTNxNa	-	-	*tetBE, aac(6')-Ib-cr*	large plasmid	
E16 (O1)	ACeCfChTCoNxNaCx	*dfrA17, aadA5*	-	*catA1, tetB*	Chromosome	
E17 (O146)	ACeGKCxChTCoSNxNa	*dfrA17, aadA5, aadA1*	*dfrA1, sat1, aadA1*	bla_{TEM}, *catA1, tetB, strA*	Chromosome, large plasmid	
AV185 (O1)	ACoCfCxKTNxSNaCtTz	-	*dfrA1, sat1, aadA1*	*catA1, tetAB, strA, aphA1-Ia, aac(6')-Ib-cr*, $bla_{CTX-M-3}$		large plasmid (~90kb)
AV189 (O25)	ACeGCfKCiCxTNxNaCtTz	-	-	*tetA, aac(6')-Ib-cr*, $bla_{CTX-M-3}$		large plasmid (~83kb)
AV193 (ONT)	ACeCoCxNxNaGKTCtTz	-	-	bla_{TEM}, *catA1, strA, tetABE, aphA1-Ia, aac(6')-Ib-cr*, $bla_{CTX-M-3}$		large plasmid (~83kb)
AV195 (O1)	ACeCo	-	-	-		

ONT, untypable; A, ampicillin; Ch, chloramphenicol; Co, co-trimoxazole; G, gentamicin; Ne, neomycin; T, tetracycline; S, streptomycin; Na, nalidixic acid; Ce, cephalothin; Am, amikacin; Cf, ceftazidime; K, kanamycin; Ci, ceftriaxone; Cx, ciprofloxacin; Nx, norfloxacin; Ct, cefotaxime; Tz, ceftazidime

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 *Xba*I PFGE patterns indicate that most of the ETEC strains in this study are distinct clones[2]. 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 rare[25]. As reported with other pathogens, use of fluoroquinolones in India may have progressively favoured emergence of MDR-ETEC strains[4-6]. 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]			
			Cx		Nx	
	GyrA	ParC	Before addition of CCCP	After addition of CCCP	Before addition of CCCP	After addition of 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→I, A108→V	0.067 ± 0.003	0.089 ± 0.0025	0.166 ± 0.003	0.461 ± 0.0021
E4	S83→L, D87→N	S80→I, A108→V	ND	ND	ND	ND
E7	S83→L, D87→N	S80→I, A108→V	0.014 ± 0.0013	0.020 ± 0.0019	0.043 ± 0.0010	0.047 ± 0.0022
E9	S83→L, D87→N	S80→I, A108→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→I, A108→V	ND	ND	ND	ND
E16	S83→L, D87→N	S80→I, A108→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 *m*-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 antibiotic[26]. 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* strains[28]. 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 alleles[7,8].

Although *E. coli* has shown to have intrinsic proton-dependent multidrug-resistant efflux pump systems, the specific activity of any one pump in its natural state and level has not been established[29]. 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 pumps[30], 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 cassettes[31]. 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 streptomycin[33]. 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 *intI2* 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 integron[20].

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 strains[22]. 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 $bla_{CTX\text{-}M\text{-}3}$ genes for bla_{TEM}, which were widely distributed in ETEC[22]. Due to co-selection, bla_{TEM} was found in association with the *catA1* in most of the ETEC strains[22]. 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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