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Molecular epidemiology of diarrhoeagenic *Escherichia coli* associated with sporadic cases and outbreaks of diarrhoea between 2000 and 2001 in India

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Abstract. Diarrhoeal infection caused by *Escherichia coli* is common in India with occasional outbreaks. However, association of different pathotypes of diarrhoeagenic *E. coli* (DEC) with the disease and its phenotypic and genotypic characteristics are not fully demonstrated. In this study, *E. coli* strains from sporadic cases and outbreaks of diarrhoea during 2000–2001 were confirmed as DEC by polymerase chain reaction (PCR) targeting the specific virulence genes. DEC represented by enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAggEC) were mostly belonged to O serogroups 25, 86a, 114 and 146. The gene a*stA* was frequently detected among ETEC and EAggEC than EPEC. After initial screening of 200 DEC strains with serology and antibiotic susceptibility test, 32 strains representing ETEC, EPEC, and EAggEC isolated from different areas of India were included in the pulsed-field gel electrophoresis (PFGE) analysis. Using the PFGE results, the hierarchical representation of different linkage levels between the DEC strains were determined by unweighed pair-group arithmetic mean (UPGAMA) method. Except for few strains, clonotyping by PFGE revealed no correlation between pathotypes and serogroups as well as the place of isolation of the DEC strains. The prevailing clonal diversity among the different categories of DEC strains suggests that the pathotypes of DEC belonged to diverse clones.

Key words: Diarrhoeagenic *E. coli* (DEC), Pathotypes, Pulsed-field gel electrophoresis (PFGE), Serogroup, Virulence genes

Introduction

Diarrhoeal diseases are endemic in many regions of Asia and are the leading cause of high degree of morbidity and mortality [1], which contributes to the deaths of 3.3 to 6 million children annually [2]. Enteric bacteria comprise the major etiologic agents of sporadic and epidemic diarrhoea both in children and adults. Detection of etiological agents of diarrhoea is important for therapeutic aspects and for implementing appropriate control strategies. In developing countries, the bacterial pathogen most commonly associated with endemic form of diarrhoea is diarrhoeagenic *Escherichia coli* (DEC). DEC belongs to different categories of pathotypes, which are classified based on their distinct clinical features, virulence mechanisms and serotypes. Currently, five distinct pathotypes of DEC are recognized: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) [3]. Serogrouping was one of the widely used methods for identifying the DEC strains [4]. Epidemiological investigations based on serogrouping are not always indicative in tracing genetic relatedness among different bacterial strains [5, 6].

Polymerase chain reaction (PCR) based typing of DEC strains have recently been developed that was found to be effective for rapid grouping of strains [7]. Several epidemiological studies reported outbreaks caused by ETEC [8, 9], EPEC [10, 11] and EAggEC [12]. Detection of clonality in DEC is a useful approach as it gives information such as source of contamination, nature of strains in the population etc. In this context, molecular epidemiology compares the genetic profiles of DEC. Early studies with multilocus enzyme electrophoresis (MLEE) to investigate the genetic relatedness of *E. coli* led to the conclusion that these organisms are essentially clonal with infrequent recombination events [13, 14].

The present study was undertaken to investigate the clonal relationship among strains identified

as ETEC, EPEC and EAggEC isolated from different areas of India, using a highly discriminatory pulsed-field gel electrophoresis (PFGE) technique.

Materials and methods

Surveillance, virulence gene detection and serology

Stool specimens collected between 2000 and 2001 from diarrhoeal patients at Infectious Diseases Hospital, Calcutta (eastern India), St. John's Medical College, Bangalore (south central India), and Kasturba Medical College, Manipal (south western India) were analyzed for the detection of DEC. In addition, DEC strains from Ahmedabad (northwestern India) and Chhattisgarh (central India) were also included in this study when there were outbreaks of diarrhoea during 2000 and 2001 respectively. Stool samples or rectal swabs were directly streaked on MacConkey agar (Difco, Detroit, USA) to obtain isolated *E. coli* colonies. The identity of these strains as *E. coli* were confirmed by different biochemical tests following standard procedure [15]. In addition, other enteric pathogens namely, *Shigella* spp., *Salmonella* spp., *Vibrio* spp. and *Aeromonas* spp. were also sought using standard culture techniques for each stool specimen [15].

Three *E. coli* colonies per specimen were further characterized by pathotype specific, virulence gene targeted PCR assays. Initial screening of *E. coli* strains was performed either in simplex or multiplex PCR with specific primer pairs (Table 1). A multiplex PCR assay was applied for the detection of EAgg, *astA* and *stx1* and *stx2* genes. Whilst, simplex PCR was performed for the *elt*, *est* and *eae* genes. These primers were shown to be specific and sensitive as compared with bioassays for the classification of DEC strains to different pathotypes [16–22]. PCR amplification was performed in a final reaction volume of 25 µl containing 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 50 mM KCl, 10 mM Tris–HCl, (pH 8.3), 1.5 mM $MgCl_2$ and 1.25 units of rTaq DNA polymerase (Takara Shuzo, Otsu, Japan). Template DNA was prepared from whole-cell lysate of isolated bacterial colonies by

Table 1. PCR primer sequences and conditions used for the detection of genes specific for diarrhoeagenic *E. coli* strains

Types of PCR	*E. coli* pathotype	Target gene or encoding region	Primer sequence (5′–3′)	Amplicon size (bp)	PCR conditions[a]	Reference
Simplex	ETEC	*elt*	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	450	94 °C 1.0 min 55 °C 1.5 min 72 °C 1.5 min	18
		est	ATTTTTA/CTTTCTGTATTA/GTCTT CACCCGGTACAA/GGCAGGATT	190	94 °C 1.0 min 55 °C 1.5 min 72 °C 1.5 min	18
	EPEC	*eae*	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCCTCTGC	454	94 °C 1.0 min 55 °C 1.5 min 72 °C 1.5 min	19
		bfpA	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	324	94 °C 1.0 min 56 °C 1.5 min 72 °C 1.5 min	20
		EAF	CAGGGTAAAAGAAGATGATAA TATGGGGACCATGTATTATCA	397	94 °C 1.0 min 60 °C 1.5 min 72 °C 1.5 min	21
Multiplex	EHEC	*stx1*	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	350	94 °C 1.0 min 55 °C 1.0 min 72 °C 1.0 min	22
		stx2	ATCAGTCGTCACTCACTGGT CTGCTGTCACAGTGACAAA	110		
	EAggEC	EAgg	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	94 °C 1.0 min 53 °C 1.0 min 72 °C 1.0 min	17
		astA	CACAGTATATCCGAAGGC CGAGTGACGGCTTTGTAG	94		16

[a] 30 cycles consisting of denaturation, annealing and extension.

boiling in a water bath for 10 min and snap cooling on ice and 2.5 μl of this template was added to the reaction mixture. Amplifications were performed in an automated thermocycler (Perkin–Elmer, Applied Biosystems, CA, USA) for 30 cycles following the specified conditions indicated in Table 1. Amplicons obtained in PCR assay were electrophoresed onto 1.5% agarose gels (Seakem, Rockland, USA), stained with ethidium bromide and the gel image was captured digitally using gel documentation system (Gel Doc 2000, Bio-Rad, CA, USA).

From about 500 strains of ETEC, EPEC and EAggEC, 200 representative strains were initially selected on the basis of pathotypes as well as serogroups. These 200 DEC strains were isolated from 200 cases of acute diarrhoea in this study. Among these 200 strains, 20 strains were selected on the basis of the proportionate preponderance of serogroups that were comparable to the number of strains isolated from three different areas of India (Calcutta, Bangalore and Manipal) covering all the pathotypes. From each diarrhoea affected area, Ahmedabad and Chhattisgarh, 12 DEC strains on the basis of the matching serogroups were included in this study. The strain details are given in Table 2. Two serologically untypable DEC strains (One each from EPEC and EAggEC) from Bangalore were also included in this study. The serogroups such as O18 (St316), O26 (St658), O158 (E11), O28ac (I23), O29 (St25437), O15 (4-2) and O167 (1-2) were represented by single strain.

All the DEC strains were cultured and stocked in colonization factor antigen broth [Casamino acid, (Difco), 1%; yeast extract (Difco), 0.15%; $MgSO_4$ (Merck, Mumbai, India), 0.005% and $MnCl_2$ (Merck), 0.0005%)] and stored at −70 °C after the addition of 15% sterile glycerol (Merck).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed by disk diffusion method [23] using commercially available discs (HiMedia, Mumbai, India). Disc containing ampicillin (A, 10 μg), chloramphenicol (C, 30 μg), co-trimoxazole (Co, 25 μg), gentamycin (G, 10 μg), neomycin (N, 30 μg), tetracycline (T, 30 μg), streptomycin (S, 10 μg), nalidixic acid (Na, 30 μg), cephalothin (Ch, 30 μg), amikacin (Ak, 30 μg), ceftazidime (Ci, 10 μg), kanamycin (K, 30 μg), ceftriaxone (Ca, 30 μg), ciprofloxacin (Cf, 5 μg) and norfloxacin (Nx, 10 μg) were used. Strains considered as susceptible, intermediately resistant, or resistant to a particular antimicrobial agent based on the diameter of the inhibitory zones as a matching criteria to the manufacturer's interpretative table that followed the recommendations of the National Committee for Clinical Laboratory Standards [24]. The ATCC strains *E. coli* 25922 and *Staphylococcus aureus* 25923 were used as quality control strains.

PFGE

PFGE procedure as described by Centers for Disease Control and Prevention [25] was adopted in this study. Briefly, bacterial strains were grown overnight on Luria Broth agar plates (Difco) at 37 °C. Confluent bacterial cultures were suspended in cell suspension buffer (100 mM Tris–HCl, 100 mM EDTA; pH 8.0) and adjusted to optical density 1.0 at 610 nm. The cell suspension (200 μl) was mixed with 10 μl of proteinase K (20 mg/ml) and an equal volume of melted 1% SeaKem agarose (Rockland, Maine, USA) containing 1% sodium dodecyl sulfate. The mixture was dispensed into a sample mould (Bio-Rad, CA, USA). After solidification, the plugs were transferred to 2.0 ml microfuge tubes containing 1.5 ml cell lysis buffer (50 mM Tris–HCl; 50 mM EDTA; pH 8.0; 1% sarcosyl) containing 1.5 mg of proteinase K. Cells were incubated at 54 °C for 2 h under mild shaking to achieve complete lysis. The plugs were successively washed twice with water and four times with TE buffer (10 mM Tris–HCl; 1 mM EDTA; pH 8.0) for 15 min each at 54 °C. The agarose plugs were equilibrated in appropriate restriction enzyme buffer for 1 h at 37 °C and the embedded DNA was digested for overnight at 37 °C with 50 U of *Xba*I (Takara). Electrophoresis was performed using the autoalgorithm mode of contour clamped homogeneous electric field Mapper system (Bio-Rad) to resolve the DNA size range of 20–350 kb with 1% PFGE grade agarose (Bio-Rad) at 6 V/cm for 40 h 24 min at 14 °C. After electrophoresis, the gel was stained with ethidium bromide and the gel image was captured digitally in a gel documentation system (Gel Doc 2000, Bio-Rad). A DNA size standard (λ ladder; New England Biolabs, MA, USA) was used as the molecular size standard.

Cluster analysis

PFGE gel images were retrieved and aligned to generate a composite image containing the banding profiles of all the strains and analyzed by the Diversity Database fingerprinting software version 2.2.0 (Bio-Rad). For construction of dendrogram, bands ranging from 48.5 to 438.5 kb were considered. Comparisons of differences in the patterns of PFGE bands were made to ascertain the clonal relationship between strains. Degrees of homology were determined by Dice comparisons and clustering correlation coefficients were calculated by an unweighed pair-group method with arithmetic mean (UPGAMA). After completion of the analysis, a dendrogram showing the hierarchical representation of linkage level between the strains was drawn.

Table 2. Phenotypic and genotypic description of *E. coli* strains from outbreak and sporadic cases of diarrhoea

Strain No.	Pathotype (setting of isolation)	Place/year of isolation	Antibiogram	Serogroup	PCR results[a]						
					Virulence gene/Marker plasmid						
					elt	*est*	*eae*	*bfpA*	EAF	EAgg	*astA*
A2	EPEC, (S)	Manipal, 2000	AChTCoNaS	O114	–	–	+	+	+	–	–
AV188	EPEC, (O)	Ahmedabad, 2000	AChGCfCaKTCCiCoNaNxN	O114	–	–	+	–	–	–	–
11045	EPEC, (S)	Calcutta, 2000	AChCCoNaS	O114	–	–	+	+	+	–	–
St95[b]	EPEC, (S)	Bangalore, 2000		ONT	–	–	+	–	–	–	–
St316	EPEC, (S)	Bangalore, 2000	AChTCoS	O18	–	–	+	–	–	–	–
St658	EPEC, (S)	Bangalore, 2000	AChS	O26	–	–	+	–	–	–	+
HD172	EPEC, (S)	Bangalore, 2000	AChCoS	O6	–	–	+	–	–	–	–
E11	EPEC, (O)	Ahmedabad, 2000	AChCiCoNa	O158	–	–	+	+	+	–	–
St102	ETEC, (S)	Bangalore, 2000	AChAkCfCaKTCCiCoNaNxS	O25	–	+	–	–	–	–	+
A11	ETEC, (S)	Manipal, 2001	AChGCfCaKTCCiCoNaNxS	O25	–	+	–	–	–	–	+
13P	ETEC, (O)	Chhattisgarh, 2001	AChCaCoNa	O1	+	–	–	–	–	–	–
E4	ETEC, (O)	Ahmedabad, 2000	AChAkCaTCCiCoNaNxS	O1	+	–	–	–	–	–	+
561	ETEC, (S)	Calcutta, 2000	AChCaCoS	O8	+	–	–	–	–	–	–
E6	ETEC, (O)	Ahmedabad, 2000	AChTCCiCoNaNxS	O8	+	–	–	–	–	–	–
I23	ETEC, (S)	Manipal, 2001	AChGCfCaKTCCiCoNaNxS	O28ac	–	+	–	–	–	–	+
AV189	ETEC, (O)	Ahmedabad, 2000	AChGCfCaKTCiNxS	O25	+	–	–	–	–	–	–
7848	ETEC, (S)	Calcutta, 2000	Na	O25	–	+	–	–	–	–	+
9P	ETEC, (O)	Chhattisgarh, 2001	AChS	O114	+	–	–	–	–	–	–
8535	ETEC, (S)	Calcutta, 2000	AChCCoNaS	O114	+	–	–	–	–	–	–
16P	ETEC, (O)	Chhattisgarh, 2001	AChCo	O146	+	+	–	–	–	–	–
E2	ETEC, (O)	Ahmedabad, 2000	AChCaTCCiCoNaNxS	O146	+	–	–	–	–	–	–
St25437[b]	ETEC, (S)	Bangalore, 2000		O29	+	–	–	–	–	–	–
4-2	ETEC, (O)	Chhattisgarh, 2001	AChCaTCCoS	O15	+	–	–	–	–	–	–
14ii	EAggEC, (O)	Chhattisgarh, 2001	ACoNa	O146	–	–	–	–	–	+	–
1-2	EAggEC, (O)	Chhattisgarh, 2001	AChGCaKTCoNa	O167	–	–	–	–	–	+	–
St671	EAggEC, (S)	Bangalore, 2000	AN	O6	–	–	–	–	–	+	–
8218	EAggEC, (S)	Calcutta, 2000	AChTCNa	O128	–	–	–	–	–	+	–
I-4	EAggEC, (S)	Manipal, 2001	ACh	O128	–	–	–	–	–	+	+
7838	EAggEC, (S)	Calcutta, 2000	AChCfTCCoNaNx	O86a	–	–	–	–	–	+	–
I-14	EAggEC, (S)	Manipal, 2001	AChCaTCoNaS	O86a	–	–	–	–	–	+	+
I-16	EAggEC, (S)	Manipal, 2001	AChTCoNa	O86a	–	–	–	–	–	+	+
St545	EAggEC, (S)	Bangalore, 2000	AChTCCoNaNxS	ONT	–	–	–	–	–	+	–

Abbreviations: ONT, not typable; S, strains from sporadic diarrhoea cases; O, strains from outbreaks.

[a] None of the *E. coli* strains yielded positive amplicons for *stx1* and *stx2*.

[b] Sensitive to all tested antibiotics.

Results

Thirty-two strains belonging to different DEC pathotypes isolated during the period from 2000 to 2001 from five widespread areas of India from sporadic cases and outbreaks were analyzed. The DEC strains included in this study were sole pathogens and no other enteric pathogen could be detected from the stool specimens. Inclusion of higher number of ETEC as compared to EPEC and EAggEC did not reflect the actual prevalence of this pathotype. This was due to the addition of four ETEC strains among six strains from Ahmedabad, where an outbreak was caused by the ETEC along with the *Vibrio cholerae* O1 and O139 serogroups [9]. Analysis for the presence of EPEC Adherence Factor (EAF) plasmid and *bfpA* gene revealed that three out of eight *eae* harbouring strains were typical EPEC [26] as they harboured both the EAF plasmid and *bfpA* (Table 2). Among the 15 ETEC strains, 10 carried the *elt*, while 4 had *est* and 1 strain had both the genes. Except for one strain (16P), which harboured both *elt* and *est* from Chhattisgarh, all the ETEC strains from Ahmedabad and Chhattisgarh were found to harbour *elt*. Enteroaggregative stable toxin (EAST1) gene (*astA*) was found mostly in *est* harbouring ETEC strains (Table 2). Of the nine EAggEC strains having EAgg plasmid, three were shown to harbour *astA*.

Serogroups O114, O25, O146, and O86a were the most common (Table 2) and represent a true picture of the distribution of these serogroups among our large collection of DEC strains (data not shown). However, distribution of particular serogroups to a specific pathotype had not been noticed in this study. Antibiotic resistance profiles against 15 antimicrobial agents revealed that all but 3 were resistant to ampicillin and cephalothin. For certain antibiotics, majority of the strains tend to reflect an area specific resistance. For e.g., DEC strains isolated from both Ahmedabad and Chhattisgarh outbreaks were resistant to co-trimoxazole, nalidixic acid, ampicillin and cephalothin, the Calcutta and Manipal strains were resistant to nalidixic acid and Bangalore strains showed resistance to streptomycin (Table 2).

In the PFGE, majority of the strains belonging to the same serogroup/pathotype displayed no similar banding profiles (Figure 1). Dendrogram derived from PFGE banding patterns was shown to be useful in the detection of clonal relatedness among DEC and to estimate the relationships among same/different serogroups. The UPGAMA method based dendrogram showed that majority of the DEC strains had no clonal relatedness (Figure 2). In addition, there is a lack of clustering among the strains of different geographic origin or strains belonging to the same serogroup. However, as shown in Figure 2, the DEC strains in cluster A and B comprised EAggEC (three out of four) and EPEC (four out of seven) respectively. These strains had different geographical origin and belongs to diverse serogroups.

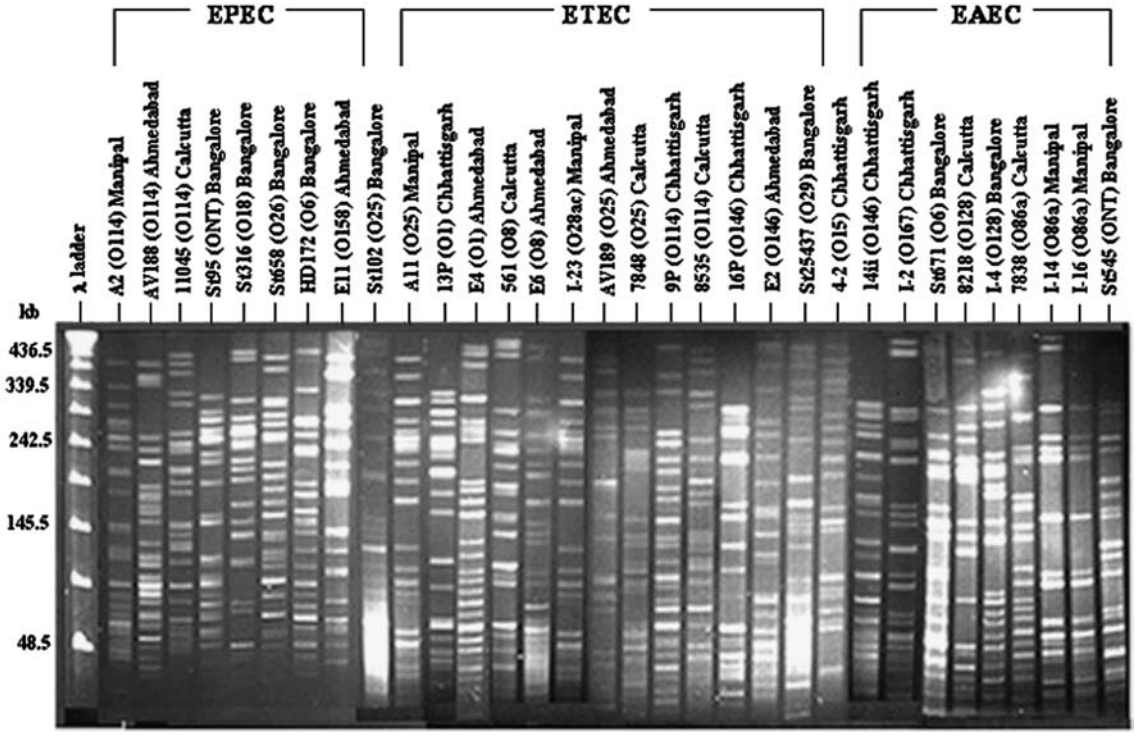

Figure 1. *XbaI* restricted PFGE profiles of the 32 diarrhoeagenic *E. coli* strains from two outbreak and three sporadic cases. Strain number, serogroup, and different areas are indicated above the each lane. The bacteriophage lambda (λ) DNA ladder, standard for PFGE (New England Biolabs) was used as DNA molecular size marker (kilo base pairs).

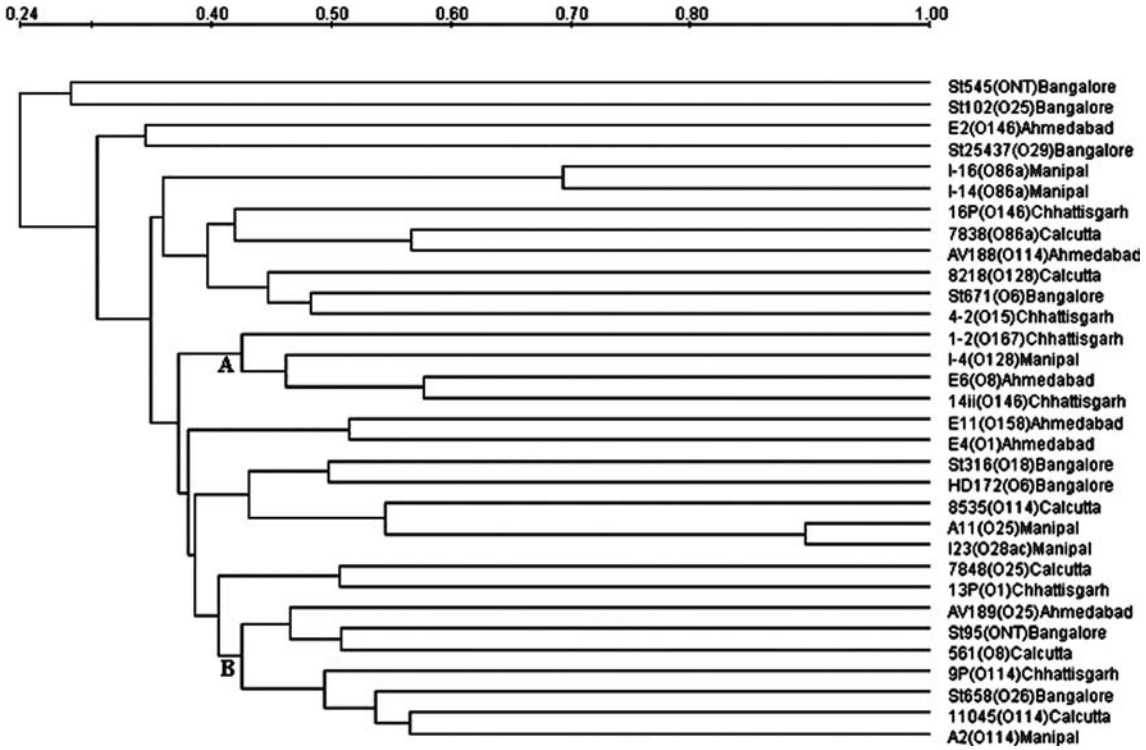

Figure 2. Dendrogram derived from PFGE profiles of the DEC strains with the Diversity Database fingerprinting software version 2.2.0 (Bio-Rad). The similarity matrix was calculated by Dice comparisons and clustering correlation coefficients were determined by an Unweighed pair-group method with arithmetic mean (UPGAMA). The similarity scale is shown above the dendrogram.

Discussion

We have tested the DEC strains for various virulence genes by PCR and serotyped the O antigen. In this study, the DEC strains associated with outbreak and sporadic diarrhoea cases belonged to 15 different serogroups and showed distinct antibiotic resistant profiles. Difference in PFGE patterns helped us to demonstrate the variability among DEC strains isolated from outbreak and sporadic cases. Considering the result of antibiotic resistant patterns, virulence gene marker and PFGE profiles, the two ETEC strains from Manipal (A11 and I23) belongs to the same clone. Strains belonged to same serogroup, however had different PFGE patterns and were resided relatively far from each other in the dendrogram, except in case of the strains of serogroups O86a (I-14 and I-16) and O114 (A2, 11045 and 9P). This is consistent with the previous report based on the MLEE patterns, which showed that *E. coli* strains belonging to the same serogroup that did not necessarily fall into the same cluster nor were the clones belonging to the same disease category [27]. Our study revealed that strains of the same pathotype of DEC are not monophyletic, i.e., not confined to a single cluster in the dendrogram. However, two strains each from ETEC (A11, serogroup O25 and I23, serogroup O28ac) and EAggEC (I-14 and I-16, serogroup O86a) pathotypes from Manipal were closely related according the clonal criteria [28] as they showed difference with less than two bands in the PFGE and shared more than 50% similarity in the dendrogram. These two ETEC strains though they belong to different serogroups are placed in the same cluster. Such a trend has been observed previously among ETEC strains isolated from an outbreak [9] and among pandemic *Vibrio parahaemolyticus* strains [29]. To our knowledge, this is the first report regarding clonal analysis employing molecular approach on DEC strains involved in sporadic and outbreak-related diarrhoea in India. Since the representative DEC strains were originally isolated from patients with diarrhoeal disease in widely separated areas, we infer that they individually mark widespread clones. From the observed set of strains it could be inferred that the DEC strains exhibited high degree of heterogeneity in genetic make up. However, prospective molecular epidemiological studies in several locations are required before arriving to any conclusion.

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