ORIGINAL ARTICLE

Role of diarrhoeagenic *Escherichia coli* in children <5 years of age hospitalised for acute/persistent diarrhoea at a tertiary care hospital in Lucknow

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ABSTRACT

Background and Objectives: Diarrhoea is one of the leading causes of morbidity and mortality among children under 5 years. Study was aimed to evaluate the role of different categories of diarrhoeagenic *Escherichia coli* (DEC) in children <5 years of age hospitalised for acute/persistent diarrhoea, by multiplex polymerase chain reaction assay for simultaneous detection of five categories of DEC using eight virulent genes and antibiotic susceptibility pattern of DEC. **Materials and Methods:** Faecal samples from 355 children with and 150 children without diarrhoea were collected from 2011 to 2013 from King George Medical University and Balrampur Hospital of Lucknow district, Uttar Pradesh, India and were tested for enteric pathogens using conventional diagnostic methods and molecular methods. **Results:** DEC was detected in 152 (42.81%) children with and 14 (9.33%) in children without diarrhoea respectively. The most common pathotype was enteroaggregative *E. coli* (EAEC) in cases and controls (16.05% and 5.33%), followed by enteropathogenic *E. coli* (12.67% and 2.66%), enterotoxigenic *E. coli* (8.73% and 1.33%), enteroinvasive *E. coli* (5.35%/and nil) in cases and controls respectively, enterohaemorrhagic *E. coli* was not detected in any of the diarrhoeal samples. DEC isolates showed a low rate of sensitivity to Ampicillin (07.89%), Amoxycillin-Clavulanic acid (06.57%), Trimethoprim/Sulphamethaxazole (20.39%), Nalidixic acid (24.34%,), Ciprofloxacin (34.34%), Ceftriaxone (23.68%)), Chloramphenicol (33.55%) and Cefoxitin (30.26%) while they were more sensitive to Amikacin (65.78% sensitive), Piperacillin/Tazobactam (61.84% sensitive) and Gentamicin (61.84% sensitive). **Interpretation and Conclusions:** DEC strains are a significant cause of diarrhoea in children. EAEC was the most frequent pathotype in the study. The high level of antimicrobial resistance in our study raises a broader discussion about the indiscriminate use or misuse of antibiotics and the risks of empirical antibiotic therapy in children of a very young age.

Key words: Children, diarrhoeagenic *Escherichia coli*, enteroaggregative *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, multiplex polymerase chain reaction, pathotype

INTRODUCTION

Escherichia coli is one of the predominant species in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has emerged that causes diarrhoeal disease in humans, referred to as diarrhoeagenic *E. coli* (DEC).[1] It is one of the most common childhood illnesses, with every child under 5 years of age in the developing world experiencing around three episodes of diarrhoea per year. A wide range of viruses, bacteria and parasites can cause the diarrhoea. Among the bacterial pathogens, *E. coli* is an important aetiologic agent of childhood diarrhoea and represents a major public health problem in developing countries. *E. coli* is one of the members of the family *Enterobacteriaceae*, which resides as a commensal flora in the intestine of animals and humans.[2] The bacterial pathogen most commonly associated with endemic form of diarrhoea is *E. coli*.[3] Among children below 5 years of age, DEC is the most important enteric pathogens and are responsible for 30-40% of all the diarrhoeal episodes in developing countries,[4] Identification of DEC strains requires that these organisms be differentiated from non-pathogenic members that constitute normal intestinal flora. Molecular characterisation of DEC is based on the presence of different chromosomal or plasmid encoded virulence genes that are absent in commensal *E. coli*.[5] DEC strains can be divided into six main categories enteropathogenic *E. coli* (EPEC), enterotoxogenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC).[6] Although DEC is of public health relevance, they are not routinely sought as enteric pathogen in

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clinical laboratories worldwide, thus their importance in community acquired diarrhoea is generally unknown particularly in the area of endemicity. The aim of the present study is the use of multiplex polymerase chain reaction (PCR) to investigate the prevalence and role of the diarrhoeagenic *E. coli* in children <5 years suffering with acute and persistent diarrhea.

Aims

1. To evaluate the role of different categories of DEC in children <5 years of age hospitalised for acute/persistent diarrhoea, by developing a multiplex PCR assay as a rapid diagnostic tool for simultaneous detection of five categories of DEC using eight virulent genes.
2. To determine the antibiotic susceptibility pattern of DEC.

MATERIALS AND METHODS

Children aged <5 years admitted to the paediatrics department with complaints of diarrhoea in King George Medical University (KGMU) and Balrampur Hospital in Lucknow, Uttar Pradesh, India from May 2011 to May 2013, were the cases. Children were enrolled in the study if they had diarrhoea characterised by the passage of three or more loose or liquid stools per day or more frequently than is normal for the individual. Acute watery diarrhoea lasts for several hours or days, and includes cholera; acute bloody diarrhoea is also called dysentery, and persistent diarrhoea was that, which lasted for 14 days or longer.[7] Control subjects of the same age group without complaints of diarrhoea were selected from the outpatient department (OPD) of paediatrics of KGMU, Lucknow, Uttar Pradesh, India. Control subjects included children presenting to paediatrics OPD for routine health check-up and minor illnesses not including diarrhoea.

Sample collection

Fresh stool specimens were collected in sterile, dry, leak proof stool container/tube and transported to laboratory as soon as possible. Cary-Blair transport medium was used for collection in case of anticipated delay of more than 1 h. After collection, the stool specimens were grossly examined for characteristics such as colour, consistency (watery, semi-formed, and formed), the presence of mucous and visible blood. Rectal specimens were taken with sterile cotton tipped swabs from those children from whom stool specimens could not be obtained at the time of collection. The sample collection was performed with the consent of the children's parent or guardian and was approved by the bioethical committee of the faculty of Medical Sciences, King George's Medical University, Lucknow, Uttar Pradesh, India. Both patients and controls were not on antibiotics in the week preceding sampling. Data was noted on a standardised questionnaire for each patient. This included demographic characteristics (age and sex), clinical history (case definitions), past and family history, epidemiological and anthropometric data.

Microbiological Methods

Specimen storage

Specimen was processed immediately, and an aliquot was stored at −20°C.

Culture and Identification of isolates

All stool specimens of children suffering from diarrhoea were cultured on MacConkey agar for the selection of *E. coli* and on other media, such as deoxycholate citrate agar (for the selection of *Shigella* and *Salmonella*), thiosulphate-citrate-bile salt-sucrose agar (for selection of Vibrio cholerae) with overnight incubation at 37°C. Each culture isolate was identified phenotypically using standard biochemical tests.[8]

Antimicrobial susceptibility assay

Antimicrobial susceptibility testing was done by disk diffusion method of Kirby-Bauer.[9] The antimicrobial agents tested were Ampicillin 10 mcg, Amikacin 30 mcg, Chloramphenicol 30 mcg, Gentamycin 10 mcg, Nalidixic acid 30 mcg, Ciprofloxacin 5 mcg, Ceftriaxone 30 mcg, Cefoxitin 30 mcg, Co-amoxiclav 30 mcg, Piperacillin/Tazobactam 100/10 mcg, Trimethoprim/Sulphamethoxazole 25 mcg.[10] *E. coli* ATCC 35218 and ATCC 25922 were used as standard strains.

Stocks of bacterial strains

The *E. coli* isolates were maintained in 1% nutrient agar stab slopes and stored in the dark.

Multiplex polymerase chain reaction methodology

We designed a multiplex PCR for the detection of five categories of DEC. This method proved specific and rapid in detecting virulence genes of DEC with some modification.[11]

Bacterial strains

The reference strains were used for standardisation of multiplex PCR assays provided by National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India. Standard strains used are as follows: Strain 1571 (ETEC); serotype 0115, strain 11044 (EPEC); serotype 0114, strain IDH 2974 (EAEC); 015, strain VT3 (STEC) serotype 0157:H7, strain H7 (EIEC).

Preparation of DNA templates for polymerase chain reaction

A smear of bacteria from the first area of MacConkey plate was suspended in 50 μl of deionised water. The bacterial

suspension was boiled for 10 min. at 95°C followed by centrifugation at 10,000 ×*g* for 10 min to pellet the cell debris. The supernatant was then used as the DNA template for PCR.

The multiplex polymerase chain reaction assay

The sequences of the primers selected for use in the amplification were completely matched with the sequences of the corresponding genes of EAEC, EHEC, EIEC, EPEC, and ETEC in the GenBank and EMBL database libraries. The minimum criteria for determination of diarrhoeagenic *E. coli* was defined as follows: The presence of eltB and/or estA for ETEC, the presence of vt1 and/or vt2 for EHEC, the presence of bfpA and eaeA for typical EPEC but the presence of only eaeA for atypical EPEC, presence of ial for EIEC and *Shigella*, and the presence of pCVD for EAEC. First, multiplex PCR was performed with reference stains of diarrhoeagenic *E. coli* from pure cultures.

Samples were tested by the multiplex PCR with the target sequence of each of the five different categories of DEC [Figure 1]. The DNA templates of *E. coli* isolates were subjected to multiplex PCR with specific primers as described previously for the detection of the following virulence markers: EaeA (structural gene for intimin of EPEC and EHEC), bfpA (structural gene for the bundle-forming pilus of EPEC), vt1 and/or vt2 (Shiga toxins 1 and 2 of EHEC), eltB and/or estA (enterotoxins of ETEC), ial (invasion-associated locus of the invasion plasmid found in EIEC and *Shigella*) and pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC) [Table 1]. To confirm, subculture from original plate was done, then PCR was done by taking five to eight colonies with typical *E. coli* morphology from the subculture plate and each colony was tested independently by PCR with a primer specific for a suspected DEC.

Polymerase chain reactions were performed in a 25 μl reaction mixture. Each reaction mixture consisted of 2 μl bacterial lysate, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 2.5 U pure Taq DNA polymerase and 0.2 μM each primer, except for primer VT1, which used at a concentration of 0.4 μM (determined as the optimal concentration after standardizing the PCR assay). Amplification was carried out in thermal cycler (Bio-Rad Laboratories), with the following thermocycling conditions: 96°C for 4 min; 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min; and a final 7 min extension at 72°C. A PCR product (10 μl) was evaluated on a 1.5% (w/v) agarose gel (BioViz) at 120 mV for 30 min. A molecular marker was run concurrently. The DNA bands were visualised and photographed under UV light after the gel had been stained with ethidium bromide.

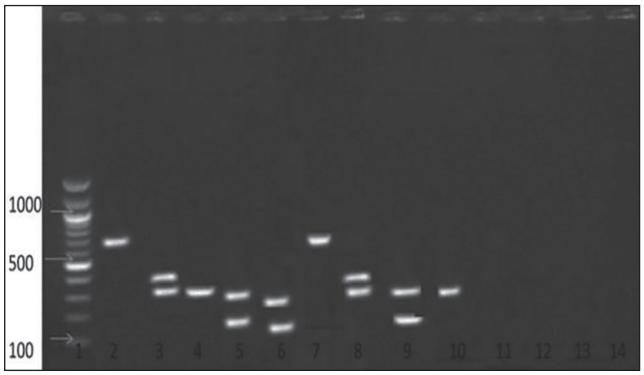

Figure 1: Multiplex polymerase chain reaction of reference strain and clinical samples

Table 1: Primers used in the multiplex PCR for the detection of DEC types

Target gene	Primer	Primer sequences (5′→3′)	Fragment size (bp)	GenBank accession number
eltB	LT	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	322	S60731
estA	ST	GTCAAACCAGTA(G/A)GGTCTTCAAAA	147	M34916
vt1	VT1	CCCGGTACA(G/A)GGAGGATTACAACA GAAGAGTCCGTGGGATTAC	130	AF461172
vt2	VT2	AGCGATGCAGCTATTAATAA ACCGTTTTTCAGATTTT(G/A)CACATA	298	AY143337
eaeA	Eae	TACACAGGAGCAGTTTCAGACAGT CACACGAATAAACTGACTAAAATG	376	AE005595
bfpA	bfpA	AAAAACGCTGACCCGCACCTAAAT TTCTTGGTGCTTGCGTGTCTTTT	367	U27184
Ial	SHIG	TTTTGTTTGTTGTATCTTTGTAA CTGGTAGGTATGGTGAGG	320	AY167049
pCVD432	EA	CCAGGCCAACAATTATTTCC CTGGCGAAAGACTGTATCAT AAATGTATAGAAATCCGCTGTT	630	X81423

DEC: Diarrhoeagenic *E. coli*, PCR: Polymerase chain reaction, *E. coli*: *Escherichia coli*

Statistical analysis used

Z-test was used to determine the statistical significance of the data. The $P < 0.05$ was considered as statistically significant.

RESULTS

Samples were collected from 355 children <5 years with diarrhoea and 150 children without diarrhoea. Detection of DEC was carried out by multiplex PCR assay. In this study, males (57.7%), predominated and 63.3% of children were <2 year of age. The average number of days since onset of diarrhoea was 5.34 ± 4.64. *E. coli* (81.12%) was the most common enteropathogen in this study. DEC was detected in 152 (42.81%) children with diarrhoea and in 14 (9.33%) children without diarrhoea. The most common pathotype was EAEC (16.05%/5.33%), followed by EPEC (12.67%/2.66%), ETEC (8.73%/1.33%), EIEC (5.35%/nil) in cases and control respectively, EHEC was not detected in any of the diarrhoeal samples. Fever was the commonest feature of diarrhoea, seen in 92.10%, followed by vomiting and abdominal cramps in 53.28% and 4.60% of in DEC cases, respectively. Mucus or blood was present, 3.28% or 1.97% in their stools respectively. Dehydration in DEC type was seen, 70 cases (46.05%) were mild, 37 (24.34%) moderate, 32 (21.05%) severe and no dehydration was seen in 13 (8.55%) cases [Table 2]. The DEC isolates showed low rate of sensitivity to Ampicillin (07.89%) followed by Amoxiclav (06.57%), Trimethoprim/Sulphamethoxazole (20.39%), Nalidixic acid (24.34%), Ciprofloxacin (34.34%), Ceftriaxone (23.68%), and Cefoxitin (30.26%) [Table 3]. Prevalence of DEC among the children with and without diarrhoea was estimated by applying the Z-test and EAEC, atypical EPEC, ETEC was significantly associated with diarrhoea ($P < 0.05$) [Table 4].

DISCUSSION

The prevalence and other epidemiological characteristics of DEC as aetiologic agents of diarrhea vary globally from region to region, and even between and within countries in the identical geographic location.[12] In this Study, 16.05% EAEC strains, were found to be the most

Table 2: Clinical symptoms in children with diarrhoea with DEC pathotype

Clinical properties	EAEC (57) (%)	EPEC (45) (%)	ETEC (31) (%)	EIEC (19) (%)	EHEC (0) (%)
Symptoms					
Fever	53 (92.98)	41 (91.11)	30 (96.77)	16 (84.21)	0 (00.00)
Vomiting	17 (29.82)	37 (82.22)	25 (80.64)	2 (10.52)	0 (00.00)
Abdominal pain	3 (5.26)	2 (4.44)	1 (3.22)	1 (5.26)	0 (00.00)
Stool properties					
Watery	23 (40.35)	37 (82.22)	29 (93.54)	11 (57.89)	0 (00.00)
Semi—formed	34 (59.64)	8 (17.77)	2 (6.45)	8 (42.10)	0 (00.00)
Formed	—	—	—	—	—
Mucous	1 (1.75)	2 (4.44)	—	2 (10.52)	—
Blood	—	1 (2.22)	—	2 (10.52)	—
Dehydration					
Mild	25 (43.85)	19 (42.22)	15 (48.38)	11 (57.89)	0 (00.00)
Moderate	13 (22.80)	11 (24.44)	10 (32.25)	3 (15.78)	0 (00.00)
Severe	15 (26.31)	9 (20.00)	6 (19.35)	2 (10.52)	0 (00.00)
None	4 (7.01)	6 (13.33)	0 (00.00)	3 (15.78)	0 (00.00)

DEC: Diarrhoeagenic *E. coli*, EAEC: Enteroaggregative *E. coli*, EPEC: Enteropathogenic *E. coli*, ETEC: Enterotoxogenic *E. coli*, EIEC: Enteroinvasive *E. coli*, EHEC: Enterohaemorrhagic *E. coli*, *E. coli*: *Escherichia coli*

Table 3: Antimicrobial susceptibility pattern of DEC

Antimicrobial disc	Resistant (%)	Intermediate (%)	Sensitive (%)
Ampicilin	140 (92.10)	—	12 (07.89)
Amikacin	52 (34.21)	—	100 (65.78)
Chloremphenecol	95 (62.50)	6 (3.94)	51 (33.55)
Gentamycine	48 (31.57)	10 (06.57)	94 (61.84)
Nalidixic acid	115 (75.65)	—	37 (24.34)
Ciprofloxacin	99 (65.13)	16 (10.52)	37 (24.34)
Amoxicillin + clavulinic acid	133 (87.50)	9 (05.92)	10 (06.57)
Ceftriaxone	98 (64.47)	18 (11.84)	36 (23.68)
Cefoxitin	92 (60.52)	14 (09.21)	46 (30.26)
Piperacillin/tazobactam	51 (33.55)	7 (04.60)	94 (61.84)
Trimethoprim/Sulphamethaxazole	121 (79.60)	—	31 (20.39)

DEC: Diarrhoeagenic *E. coli*, *E. coli*: *Escherichia coli*

Table 4: Prevalence of DEC in children with and without diarrhoea

DEC type	Virulence gene	Children with diarrhoea (%)	Children without diarrhoea (%)	OR	*P* (χ^2)	95% CI
		n = 355	*n* = 150			
EAEC	pCVD432	57 (16.05)	8 (5.33)	0.0340	0.0018	1.5775-7.3072
EPEC atypical	eaeA only	36 (10.14)	4 (2.66)	4.1191	0.0083	1.4394-11.7876
EPEC typical	eaeA, bfpA	9 (2.53)	0 (00.00)	0.0274	0.0760	0.0005-1.4565
ETEC	Eltb	27 (7.60)	2 (1.33)	6.0915	0.0145	1.4297-25.9529
	estA	4 (1.12)	0 (00.00)	0.0128	0.0340	0.0002-0.7194
EIEC	Ial	19 (5.35)	0 (00.00)	0.0579	0.1572	0.0011-2.9991
EHEC	vt1	0.00	0 (00.00)	1.0000	1.0000	0.0039-255.6264
	vt2	0.00	0 (00.00)	1.0000	1.0000	0.0039-255.6264

DEC: Diarrhoeagenic *E. coli*, EAEC: Enteroaggregative *E. coli*, EPEC: Enteropathogenic *E. coli*, ETEC: Enterotoxogenic *E. coli*, EIEC: Enteroinvasive *E. coli*, EHEC: Enterohaemorrhagic *E. coli*, *E. coli*: *Escherichia coli*, OR: Odds ratio, CI: Confidence interval

Table 5: Age distribution of DEC in children with diarrhoea

Age of patient (months)	Number of cases	EAEC (%)	EPEC (%)	ETEC (%)	EIEC (%)	EHEC (%)
<6	47	8 (17.02)	14 (29.78)	3 (6.38)	0 (0.00)	0 (0.00)
6-11	86	9 (10.46)	21 (24.41)	5 (5.81)	4 (4.65)	0 (0.00)
12-23	92	9 (9.78)	6 (6.52)	16 (17.39)	3 (3.26)	0 (0.00)
>24	130	31 (23.84)	4 (3.07)	7 (5.38)	12 (9.23)	0 (0.00)

DEC: Diarrhoeagenic *E. coli*, EAEC: Enteroaggregative *E. coli*, EPEC: Enteropathogenic *E. coli*, ETEC: Enterotoxogenic *E. coli*, EIEC: Enteroinvasive *E. coli*, EHEC: Enterohaemorrhagic *E. coli*, *E. coli*: *Escherichia coli*

prevalent and may be emerging pathogen. These types of *E. coli* harbour 60-kD plasmids (pCVD432) encoding toxins and virulence factors that vary in intensity and combination worldwide.[13] The second commonest enteropathogen was EPEC (12.67%/2.66%), both in cases and control respectively. EPEC is also a very important pathogen in children with diarrhoea. EPEC infection is primarily a disease of infants younger than 2 years of age. Of the 45 EPEC, 36 (10.14%) were atypical aEPEC and 9 (2.53%) were atypical tEPEC. EPEC was more prevalent in children of age <12 months than EAEC, ETEC and EIEC, which were more prevalent in children above 12 months of the study [Table 5].

CONCLUSION

Diarrhoeagenic *E. coli* strains are a significant cause of diarrhoea in children. The use of multiplex PCR for the simultaneous detection of different virulence genes could be used as a method for rapid diagnosis and characterisation of diarrhoeagenic *E. coli* in children, and will help investigators to clarify the role of diarrhoeagenic *E. coli* in diarrheal diseases. EAEC was the most frequent pathotype in the population under study. The high level of antimicrobial resistance observed in our study raises a broader discussion about the indiscriminate use or misuse of antibiotics and the risks of empirical antibiotic therapy in children of a very young age.

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