Prevalence and molecular detection of diarrheagenic *Escherichia coli* in pediatric age group attending a tertiary care hospital in Coimbatore, Tamil Nadu

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Abstract:
Purpose: Diarrheal diseases are a major cause of morbidity and mortality among children in developing countries. Among the bacterial pathogens, diarrheagenic *E. coli* (DEC) are the most common agents causing diarrhea worldwide. The objective of this study was to determine the prevalence of DEC in stool samples from children with acute diarrhea using polymerase chain reaction (PCR). **Methods:** A total of 75 stool samples from children under the age of 15 with acute diarrhea were collected by aseptic measures for a period of one year. The selected samples were screened for *E.coli* by standard microbiological methods. Identification of pathogenic *E.coli* was based on cultural characters and standard biochemical reactions. The *E.coli* isolates were subjected for PCR analysis to classify as EPEC, EAEC, STEC and ETEC strains using their virulence marker genes. **Results:** 17 (23%) DEC isolates were identified from 75 stool samples. EPEC showed high prevalence (12%) followed by EAEC (5.33%) and STEC (4%). No ETEC strains were isolated from the population. **Conclusion:** The study showed that EPEC (12%) is the predominant strain among other DEC in causing diarrhea among children.

Key words: Acute diarrhea; Diarrheagenic *Escherichia coli*; Enteropathogenic; Polymerase chain reaction

Introduction:

Diarrheal diseases are the major cause of death in children under 5 years of age in developing countries, resulting in approximately 2.5 million deaths each year worldwide [1]. Among the bacterial pathogens, diarrheagenic *E. coli* (DEC) are most frequently implicated in cases of epidemic and endemic diarrhea [2]. However, the detection of DEC strains are difficult since these strains cannot be easily distinguished from the normal fecal flora using conventional phenotypic methods. *Escherichia coli* are the predominant facultative anaerobe of the human colonic flora. Gastrointestinal colonization occurs within hours of life and the bacteria will remain with a symbiotic relationship with the host [3]. *E.coli* remains harmlessly confined to the intestinal lumen. In the debilitated or

immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection [4]. Molecular identification and classification of DEC is established by the presence or absence of one or more specific virulence genes, which are absent in the commensal *E coli* [2]. DEC strains are classified into 6 distinct pathogenic categories (pathotypes) including Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) [1].

For many years, typical EPEC was considered to be a major cause of persistent diarrhea in infants, but recent studies indicate that atypical EPEC are more prevalent than typical EPEC in both resource‑rich and resource‑poor countries and may be an emerging pathogen. Very few studies have been performed in India to investigate the prevalence and characterization of DEC in patients with diarrhea [5, 6]. The objective of this study was to determine the prevalence of DEC in stool samples from children with acute diarrhea attending the pediatric department of Karpagam Faculty of Medical Sciences and Research, Coimbatore, Tamil Nadu.

Materials and Methods
Study population

The study was conducted for a period of one year from May 2013 to May 2014. A total of 75 stool samples from children under the age of 15 with acute gastritis were collected by aseptic measures. Diarrhea was defined by the occurrence of >3 loose stools, liquid or watery or at least 1 bloody stool in a 24 h period. Samples from patients who received antibiotics before admission or during their hospital stay were excluded from the study.

Culture and identification of bacterial isolates

Stool samples were collected in sterile plastic vials, transported to microbiology laboratory and initially screened for the presence of leukocytes, red blood cells, ova, and cysts of parasites using conventional microscopy and cultured on the same day to isolate *E. coli*. Samples were inoculated into selective enrichment medium [Lauryl sulphate tryptose broth (LSTB)] and after overnight incubation, subcultured onto Sorbitol MacConkey's agar and MacConkey's agar (Hi-Media, Mumbai, India). The culture plates were incubated at 37°C for 18‑24 hours. Lactose and non‑lactose fermenting colonies from MacConkey agar and sorbitol fermenting and non‑sorbitol fermenting colonies from sorbital agar were selected and subjected to conventional biochemical tests for identification of *E. coli* [7]. A total of 200 *E.coli* isolates (100 each from direct culture method and Enrichment medium respectively) were selected for PCR assay.

DNA extraction

DNA from stool samples was extracted using QIAamp DNA stool mini kit (Qiagn, GmbH, Hilden, Germany). All isolated *E. coli* strains (200 strains) were grown on Luria-Bertani broth (Sigma, St. Louis, MO) overnight at 37°C. Genomic DNA was extracted by a boiling method [8] and used as the target for polymerase chain reaction (PCR) assays.

PCR, primers, and products

All PCR primers and molecular reagents were purchased from Bangalore Genei, India. Each PCR assay was performed in a 30 ml reaction volume containing 3 ml of $10 \times$ PCR buffer (100 mM Tris HCl, 20 mM $MgCl_2$, 500 mM KCl), 200 mM concentrations of each of the 4‑deoxyribonucleotide triphosphates (dNTPs), 0.5 mg of each primer, and 1U *Taq* DNA polymerase (Bangalore Genei, India). The DNA samples carrying the relevant virulence gene (s) served as positive controls in each reaction. Sterile distilled water served as negative control.

Table 1: Oligonucleotide primers used in the study for detection of diarrheagenic *E.coli*

Category	Target gene	Primer pair	Sequence	
			Amplicon size (bp)	Reference
EPEC	*bfp*A	5′‑AAT GGT GCT TGC GCT TGC TGC‑3′ 5′‑GCC GCT TTA TCC AAC CTG GTA‑3′	324	9
	eaeA 5	5′‑GAC CCG GCA CAA GCA TAA GC‑3′ 5′-CCA CCT GCA GCA ACA AGA GG‑3′	384	10
EAEC	*ast*A	5′‑GCC ATC AAC ACA GTA TAT CC‑3′ 5′-GAG TGA CGG CTT TGT AGT CC-3'	106	11
STEC	*Stx*	5′‑.GAA CGA AAT AAT TTA TAT GT‑3′ 5′‑TAC TGA CAT TGT TAG TTT‑3′	900	12
	stx1	5′‑ACACTGGATGATCTCAGTGG‑3′ 5′‑CTGAATCCCCCTCCATTATG‑3′	614	13
	stx2	5′‑CCATGACAACGGACAGCAGTT‑3′ 5′‑CCTGTCAACTGAGCAGCACTTTG‑3′	779	13
	stx common	5′‑. GAGCGAAATAATTTATATGTG‑3′ 5′-TGATGATGGCAATTCAGTAT‑3′	518	14
	ehxA	5′‑TTT ACGA TAG ACT TCT CGA C‑3′ 5′‑CAC ATA TAA ATT ATT TCG CTC‑3′	116	15
ETEC	*lt*	5′‑GGCGACAGATTATACCGTGC‑3′ 5′‑CCGAAATTCTGTTATATATGTC‑3′	696	16
	st	5′‑TTAATAGCACCCGGTACAAGCACG‑3 5′‑CTTGACTCTTCAAAAGAGAAAATTAC‑3′	147	17

Detection of virulence genes associated with DEC

The following virulence associated genes of DEC were used as the targets for PCR amplification: attaching and effacing factor (*eaeA*), shiga‑like toxins (*stx*, *stx*1, *stx*2), heat labile toxin (*lt*), heat stable toxin (*st*), bundle forming pilus (*bfpA*), enteroaggregative stable toxin (*astA*), and hemolysin (*ehxA*). A total of 200 *E. coli* biochemically confirmed isolates from samples (100 isolates from direct stool samples and 100 isolates from enrichment broths) were tested by PCR for presence of various virulence genes associated with DEC. PCR was performed individually using each primer pair as previously described [9-17] (Table 1). PCR products were separated on a 2% agarose gel, stained with Ethidium bromide and photographed using a gel documentation system. *E. coli* reference strain EDL 933 was used as positive control in all PCR reactions for EPEC and STEC. *E coli* ATCC 25922 was used as negative controls in each batch of reactions.

Results

From May 2013 to May 2014 a total of 75 children with diarrhea were enrolled for this study. Of these 75 stool samples, Diarrheagenic *E.coli* was detected in 17 (23%) using PCR method (Table 2).

Table 2: Prevalence of pathogenic *E. coli* in stool samples determined by PCR

Sample	Total number tested	Total No. of DEC	Prevalence %
Direct culture	75	10	13%
Enrichment culture	75	17	23%

The most prevalent DEC type was EPEC accounting for 9(12%) cases followed by EAEC 4 (5.33%) and 3 (4%) cases of STEC (Table 3). No ETEC strains were isolated from any of the collected stool samples. Irrespective of whether a sample is positive for pathogenic *E. coli* or not by direct PCR, isolates of *E. coli* were collected from all the samples before and after enrichment. A total of 200 biochemically confirmed E.coli isolates from stool samples (100 isolates from direct stool samples and 100 isolates from enrichment broths) were tested by PCR for presence of various virulence genes associated with DEC. (Table 3).

Table 3: Prevalence of diarrheagenic *E. coli* (DEC) pathotypes in participants with acute diarrhea (*n* = 75)

DEC type		Target gene amplified	No. of samples %	
			Direct culture	Enrichment culture
EPEC	Typical	*eae* and *bfp*	0	0
	Atypical	*eae*	7 (9.33%)	9 (12%)
STEC		*stx*	1 (1.33%)	3 (4%)
EAEC		*astA*	2 (2.66%)	4 (5.33%)
ETEC		*lt, st*	0	0
Total			10 (13.33%)	17 (22.66%)

Target genes for amplification and for the detection of EPEC were *eae* and *bfp. stx* and *ehxA* genes were for used for STEC, *lt* and *st* genes were used for ETEC and *astA* gene for EAEC (Table 1 & Table 3). All EPEC isolates were positive for *eae* gene but negative for *bfp* and *stx* gene and were classified as atypical EPEC genotype (*eae+, bfp ⁻, stx ⁻*). A total of 9 (12%) stool samples were positive for *eae* gene, but negative for *bfp* and *stx* gene [Figure 1].
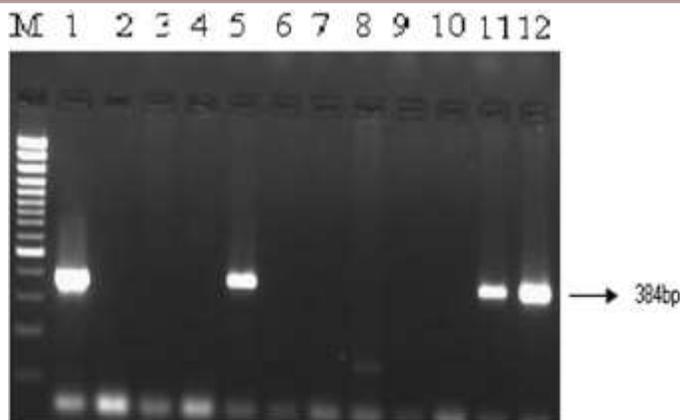

Figure 1: Direct detection of *eaeA* gene of enteropathogenic and enterohemorrhagic *E. coli* in stool samples by PCR. Lane M, 100 bp DNA ladder (Genetix); Lane 1, Reference strain EDL 933; Lane 2, Negative control strain; Lanes 3‑12, Stool 91 to Stool 100; Lanes 5, 11 and 12, Stool samples positive for *eae* genes.

STEC strains differ from EPEC genotypically in having *stx* genes. 3 (4%) isolates were positive for *stx* and were confirmed as STEC. Two sets of primers (*stx*1 & *stx*2) were used to identify the variants of STEC [13]. 4 (5.33%) isolates were positive for *astA* gene which is specific for EAEC. ETEC strains were not identified from the study group as all of them were negative for *lt* and *st* genes.

Discussion

The study was conducted to investigate the prevalence of diarrheagenic *E.coli* strains from pediatric age group with help of PCR. The study group was the children with diarrhea attending the pediatric department of Karpagam Faculty of Medical Sciences and Research, Coimbatore for a period of 1 year. The prevalence of DEC from this study population was 23% (17). The prevalence of DEC as the etiological agent of diarrhea among children varies globally from region to region depending upon the socio-economic status and other living conditions of the population.

The study showed that EPEC (12%) is the predominant strain among other DEC in causing diarrhea among children. The result correlates with the study conducted in Mangalore [18] with the prevalence of 11% for EPEC and another study from Kashmir with 7.6% prevalence [6].

A significant association of typical EPEC strains (*eae+ bfpA+ stx⁻*) with diarrhea was reported previously from developing countries [2]. In recent years, several studies have shown that atypical EPEC strains (*eae+*, *bfpA⁻* and *stx⁻*) are more prevalent than typical EPEC strains in developed countries as well as in economically poor countries including Mexico, Nicaragua, Vietnam, and Mozambique and in many other African countries. This data points towards the poor sanitary conditions in these areas. Atypical EPEC have been reported in food‑borne and water‑borne outbreaks [19].The role of atypical EPEC, causing diarrhea in children is still unclear, and future studies are warranted to investigate the epidemiology and pathogenesis of atypical EPEC strains.

STEC strains differ from EPEC in having *stx* gene. The present study showed low prevalence (4%) of STEC in pediatric diarrheal cases in Coimbatore. India has low prevalence rate of STEC [5, 6]. Two sets of primers (*stx*1 & *stx*2) were used to identify the variants of STEC. STEC has more incidence rate in the United States, Europe, Australia, South America, and parts of Asia. Prevalence of EAEC strains was 5.33%. These strains are associated with acute and persistent watery diarrhea in children, travelers and in individuals infected with HIV/AIDS [18]. The low prevalence of EPAC was reported from Brazil [20].

In this study, no ETEC strains were detected. ETEC is a ubiquitous pathogen, commonly transmitted via contaminated food and water. ETEC is a major cause of traveler's diarrhea and infantile diarrhea in resource limited countries. Previous studies from India showed varied findings ranging from 0.92% in Kashmir to 12% in Calcutta [6].

Identification of *E.coli* isolates for PCR analysis were from both direct culture plates and from the enrichment medium (Lauryl sulphate tryptose broth) after incubation. Incubation of stool samples in enrichment medium showed more positive isolates (23%) of DEC than from direct culture (13%). This could be due to two reasons. First, although viable DEC may be present in low numbers in some samples, the strains may not be cultured, and therefore, an enrichment step may be necessary for their detection. The presence of inhibitors in the stool specimens is another possibility [7]. Therefore, extraction of DNA after enrichment of the samples may have resulted in better detection rates of DEC.

The study showed the advantages of molecular assays like PCR in diagnosing DEC, thereby providing the pathogen specific treatment as some pathogens warrant antibiotic therapy while for certain DEC strains (e.g., STEC), antibiotics are not recommended due to increased risk of hemolytic uremic syndrome (HUS). This has other advantages in preventing the emergence of resistant strains and their spread. But in a developing country like India, it is difficult to ensure the availability of this facility for all and in all clinical laboratories.

Acknowledgement:

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