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Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India

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Abstract

The prevalence of diarrheagenic *Escherichia coli* (DEC) in children under 5 years was studied in children with diarrhea and controls in South India. Four polymerase chain reaction (PCR) “schemes” were used to detect genes of the 6 pathotypes of DEC. In 394 children with diarrhea, 203 (52%) DEC infections were found. Among the 198 controls, 126 (63%) DEC infections were found. Enterotoxigenic *E. coli* was the most common pathotype by multiplex PCR both in cases (58, 14.7%) and controls (47, 23.7%), followed by enteropathogenic *E. coli* seen in 10% cases and 8% of controls. Enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) were found in 4.1%, 2.0%, 1.0%, and 0.5% of cases, respectively. ETEC was found in 2.5% of controls, but EHEC, EIEC, and DAEC were not detected. Overall, no single assay worked well, but by discounting genes with a pathogenicity index of less than 1, it was possible to use the PCR assays to identify DEC in 75/394 (19%) cases and 12/198 (6.1%) controls, while mixed infection could be identified in 8/394 (2%) cases and 2/198 (1%) controls.

Keywords

Diarrheagenic *E. coli*; Multiplex PCR; Virulence genes; Adherence assays

1. Introduction

Diarrhea is one of the major causes of morbidity and mortality among children less than 5 years of age in developing countries (Kosek et al., 2003) with global mortality rates estimated to be around 1.87 million (Boschi-Pinto et al., 2008). In India, around 23% of childhood mortality has been reported due to diarrhea (Jones et al., 2006). Diarrheagenic *Escherichia coli* (DEC) are reported to be a common cause of childhood diarrhea in developing countries (Presterl et al., 2003; Rodrigues et al., 2002), with a higher incidence during the first 2 years of life (Fule and Kaundinya, 1985).

DEC are categorized into 6 major pathotypes depending on their virulence genes and include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and diffusely

adherent *E. coli* (DAEC) (Vidal et al., 2005). EAEC is characterized by the virulence factors that is present in the 60 MDa plasmid (Baudry et al., 1990), which includes aggregative adherence factor II (*aafII*) (Czeczulin et al., 1999), a heat stable toxin *ast* (Muller et al., 2007), the transcriptional activator *aggR* (Nataro et al., 1994), and a novel antiaggregation protein (dispersin) encoded by the *aap* gene (formerly known as *aspU*) (Sheikh et al., 2002). EPEC is characterized by the presence of intimin (*eae*) gene causing attachment and effacement on intestinal epithelial cells and the bundle-forming pili (*bfp*) gene (Jerse et al., 1990). Typical EPEC is characterized by the presence of both *eae* and *bfp* genes, while atypical EPEC possess the *eae* gene alone. EHEC is characterized by the presence of 2 toxin genes, *stx1* and *stx2*, for toxins that are similar to those of *Shigella dysenteriae* and, in some strains, the presence of the intimin (*eae*) gene. The presence of 1 or more heat stable (*stI* and *stII*) and labile (*ltI* and *ltII*) toxin gene characterizes ETEC strains. The invasive property of EIEC is determined by the presence of a 140-MDa virulence plasmid (*pInv*), which encodes a number of genes for invasion that includes *virf*, *ipah*, *ipal* genes, etc. (Berlutti et al., 1998). DAEC is characterized by the presence of *dae* gene, which is essential for the expression of fimbriae, a putative virulence factor that mediates adherence of this pathotype (Bilge et al., 1989). DEC are identified and differentiated from *E. coli* of the intestinal flora by the presence of these specific virulence markers that are not present in the latter (Moyo et al., 2007).

The different virulence groups of DEC can be detected by culture, biochemical reactions, serotyping, and other phenotypic assays, as well as molecular methods (Nataro and Kaper, 1998). Polymerase chain reaction (PCR) has proved to be more sensitive and specific than most conventional techniques and has been used to identify DEC in a number of studies (Al-Gallas, Bahri et al., 2007; Estrada-Garcia et al., 2009; Hien et al., 2008; Takahashi et al., 2008). Previous reports from India have documented EAEC to be the predominant pathotype found in children (Ballal and Ramamurthy, 2005; Dutta et al., 1999). In this study, we carried out characterization of the pathotypes with special emphasis on EAEC by screening stool samples of children with and without diarrhea by PCR to determine association with disease and, therefore, which assay is best applied to the detection of DEC in our region.

2. Materials and methods

2.1. Study subjects

In this study, 394 children from Vellore aged less than 5 years with acute diarrhea requiring hospitalization and rehydration for at least 6 h were recruited as cases. Diarrhea was defined as the passage of 3 watery stools in a 24-h period, and a diarrheal episode was defined as at least 1 day of diarrhea, preceded and followed by 2 or more days without diarrhea. The study was carried out between 2003 and 2006. During the same period, 198 children from a community-based surveillance study in Vellore, who did not have diarrhea in the previous month, were recruited as controls. Written informed consent was obtained from the parent or guardian of the child before recruitment, and the study was approved by the Institutional Review Board, Christian Medical College, Vellore, India. Stool samples collected from the children were aliquoted and stored as 3 vials, 1 at 4 °C and 2 at -70 °C until further use.

2.2. Stool examination for enteric pathogens

The samples were cultured on blood agar and selective media, which included MacConkey agar, xylose lysine deoxycholate agar, and thiosulfate citrate bile salt sucrose agar (TCBS) agar, and were incubated at 37 °C overnight. Samples were also inoculated in selenite F broth and alkaline peptone water for *Salmonella* and *Vibrio* spp. and subcultured onto MacConkey agar and TCBS agar, respectively, and identified by standard biochemical methods and serogrouping. For identification of EPEC, slide agglutination with antisera to

common EPEC O antigens was carried out. PCRs were also carried out to screen for rotavirus (Iturriza Gomara et al., 2002), *Campylobacter* spp. (Linton et al., 1997), and *Cryptosporidium* spp., (Xiao et al., 1999).

2.3. PCR for DEC

DNA extraction was carried out on stool samples using QIAamp DNA stool minikit (Qiagen, Valencia, CA). The extracted DNA was stored at -20°C . Initially, a DEC multiplex PCR to detect the virulence genes of all 6 categories of DEC (Vidal et al., 2005) was carried out. This was followed by 2 separate PCRs to detect *stI* (ETEC) (Osek, 2001) and *ast A* gene (EAST toxin, EAEC) (Muller et al., 2007). An additional multiplex PCR to detect different genes of EAEC (Cerna et al., 2003) was also carried out using previously published primers (Table 1). Reference strains used as positive controls during PCR were obtained from the Health Protection Agency, Colindale, London, UK, and are shown in Table 1. For the *ast* and *stI* PCR, a previously positive in-house control sample confirmed by sequencing was included for all runs. A negative control, nonpathogenic *E. coli* strain K12 was included in all batches of extraction and PCR.

2.4. Statistical analysis

Data were analyzed using STATA 10.0 for Windows (StataCorp., College Station, TX) software. Comparison between 2 groups was done using Mann–Whitney *U* test for continuous variables and χ^2 test or Fisher's exact test for categorical variables.

3. Results and discussion

In this study, PCR assays were used to detect pathotypes of *E. coli* in 394 children with diarrhea and 198 control children. The median (interquartile range [IQR]) age of the children enrolled in the study was 10 (7) months, with >90% of children less than 2 years of age. A total of 203 (52%) and 126 (63%) DEC pathotypes were found in cases and controls, respectively. In addition to this rotavirus, *Campylobacter* spp. and *Cryptosporidium* spp. were seen in 159, 17, and 28 cases and 15, 3, and 4 controls, respectively. *Vibrio cholerae* and *Shigella flexneri* were detected by stool culture in 1 case each. Coinfections with multiple putative pathogens were seen in 87 cases, making it difficult to define the etiology of gastroenteritis in these children.

In the initial screening with the DEC multiplex PCR (Table 1, Fig. 1A), for detecting the 6 categories of DEC (Vidal et al., 2005), EPEC was the most common pathotype seen in 39 (9.9%) cases and 16 (8.1%) controls, while EAEC (*aafII* gene) was found in only 3 cases. Among the EPEC, 24 (6.1%) cases were infected with typical EPEC that had both *eae* and *hfp* genes and 15 (3.8%) with atypical EPEC that had the *eae* gene alone. In controls, 5 (2.5%) were typical EPEC and 11 (5.5%) were atypical EPEC. In our setting, typical EPEC was more commonly seen in cases while atypical EPEC was seen in controls. In contrast, another study on pediatric diarrhea from Kashmir found atypical EPEC to be more common in symptomatic infections (Wani et al., 2006).

ETEC was found in 16 (4.1%) cases and 8 (4%) controls in the DEC multiplex PCR and the ETEC PCR for *stI* detected infection in an additional 6 cases and 1 control. The combination of *lt* with *stI* or *stII*, and *stII* with *stI* were seen in 1 case each, while *lt*, *stII*, and *stI* alone were seen in 6, 3, and 4 cases, respectively. In controls, *lt* alone was seen in 7 controls and *stII* was seen in 1 control. Overall, among the toxin genes, only *stI* was seen exclusively in cases. Our data differ from Bangladesh, where ETEC was found in 17.4% and 7.3% of cases and controls, respectively (Albert et al., 1999). As found in this study, other reports have

shown that the combination of more than 1 toxin genes has greater association with symptomatic infection (Albert et al., 1995; Kim et al., 1989).

EHEC, EIEC, and DAEC were detected in 8 (2%), 4 (1%), and 2 (0.5%) cases and were not detected in any controls (Table 2). Coinfections with more than 1 pathotype were seen in 15 (3.8%) cases and 7 (3.5%) controls. A recent review from India on EHEC reported that it was predominantly present in the environment and reservoirs, but it did not play role in infantile diarrhea (Khan et al., 2002). However, our finding of EHEC only in symptomatic infections, although in low numbers, suggests that this pathotype should be monitored in future in infantile diarrhea. In the case of DAEC and EIEC, the prevalence rate is too low to draw any inferences regarding significance in infantile diarrhea. However, it has been previously reported that in diffusely adhering *E. coli* strains, the *daaE* gene is not expressed frequently (Campos et al., 1999). This may explain the low level of detection of the *daaE* gene in our study.

When the additional PCRs for EAEC EAST toxin (EAEC PCR) (Muller et al., 2007) and the EAEC multiplex PCR (Fig. 1B) (Cerna et al., 2003) were taken into account, EAEC amplicons were seen in 74 (18.8%) and 58 (14.7%) of 394 cases, respectively. However, EAEC were also found to be common in controls, and the 2 amplicons were detected in 55 (27.8%) and 47 (23.7%) of 198 controls, respectively.

The high prevalence of EAST (*astA*) toxin of EAEC, in both cases and controls, indicates its lack of association with diarrhea. This is supported by previous reports that have documented the presence of this toxin in other types of pathogenic and nonpathogenic *E. coli* (Jenkins et al., 2006; Savarino et al., 1996; Zhou et al., 2002) and also in samples from both adults and children without diarrhea (Vila et al., 1998). When the individual virulence genes were examined (Table 2), among the EAEC isolates by multiplex PCR, all 3 genes (*aap*, *aggR*, and *AA*) were seen in 24 (6.1%) cases and in 6 (3%) controls, and this combination was significantly associated with diarrhea ($P < 0.001$). Earlier reports have stated that the presence of all 3 genes in EAEC is essential for pathogenesis (Cerna et al., 2003). Other studies have also shown that strains harboring *AA* plasmid are associated with more severe disease than the nonadherent strains lacking this plasmid (Czeczulin et al., 1999; Jiang et al., 2002). Five cases and no controls also had *astA* in combination with *aap*, *aggR*, and *AA*. In this study, the *aap* and *aggR* combination was seen in 19 (4.8%) cases and 10 (5.1%) controls, while the *aap* gene alone was more common in controls (29, 14.6%) than cases (13, 3.3%). Previous reports from India using adherence assays, PCR, and biofilm production have reported a high prevalence of EAEC in cases (Dutta et al., 1999; Kahali et al., 2004; Paul et al., 1994); however, our study found high rates of detection of *astA*, *aap*, and *aggR* in controls, and only the combination of *aap*, *aggR*, and *AA* was significantly associated with disease. There was no overlap between the *aafII* gene detected in the multiplex DEC PCR and the other 2 EAEC PCRs. The low number of EAEC (3) in cases identified by the *aafII* primers can be explained by a previous report, which suggests that most EAEC strains do not express the aggregative adherence factor even in the presence of the *aggR* regulon or the plasmid borne genes (Boisen et al., 2008).

When the association of the DEC pathotypes with age and clinical features were examined, there was a significant association only between lower age in months and the detection of typical EPEC (median, 5.5 months; IQR, 12.5, $P = 0.048$) and EHEC (median, 3.5 months; IQR 3.5, ($P = 0.003$). Other studies from various parts of the world have reported the role of EPEC and EHEC in infantile diarrhea (Chen and Frankel 2005; Kozub-Witkowski et al., 2008; Nataro and Kaper 1998). EAEC were found to be significantly associated with vomiting ($P = 0.03$) among the DEC (Table 3). This association has been reported in

previous studies from Switzerland where 55% of patients with EAEC had vomiting (Pabst et al., 2003) and also from Germany (Huppertz et al., 1997).

Examining the pathogenicity index (Levine 2006) of the DEC showed that typical EPEC had an index of 2.4, followed by EAEC with 2. Indices could not be calculated for EHEC, DAEC, and EIEC because there were no positives among controls, but with 2% of all cases having EHEC, this is likely to be an important pathotype in India. Overall, no single published PCR reaction was suitable for detecting pathogens over DEC carried in controls in this study. However, a multiplex assay that identifies *AA*, *aafII*, *bfp*, *eae*, *lt*, *stI*, *stII*, *stxI* and *stxII*, *virf*, *ipaH*, and *daaE*, and a classification that identified EAEC only when *aap*, *aggR*, and *AA* are all present or *aafII* alone is present, EPEC only when *bfp* and *eae* are both present and ETEC when either *stI* or *stII* or more than 1 toxin are present, would have identified DEC in 75 (19%) out of 394 cases and 12 (6%) out of 198 controls, making it more suitable for identifying pathogens in this region.

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Fig. 1.

(A) Multiplex PCR for DEC. Lane M: marker. Lane 1: EHEC—*stx1* (348 bp), *stx2* (584 bp), *eae* (482 bp). Lane 2: EPEC—*eae* (482 bp), *bfp* (300 bp). Lane 3: ETEC—*lt* (218 bp), *stII* (129 bp). Lane 4: EAEC—*aafII* (378 bp). Lane 5: DAEC—*daaE* (542 bp). Lane 6: EIEC—*virf* (618 bp), *ipah* (933 bp). Lane 7: ETEC—*stI*. (B) Multiplex PCR for EAEC. Lane M: marker. Lane 1: *aap* (310 bp). Lane 2: *aap* and *aggR* (457 bp). Lane 3: *aap*, *aggR*, and *AA* (629 bp).

Table 1

Primers for the different PCRs used in this study on DEC

PCR	Primer sequence and control strains	Product size (bp)
<i>DEC multiplex PCR</i> (Vidal et al., 2005)		
<i>EHEC</i>	E3787	348
<i>stx1</i>	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG	
<i>stx2</i>	E32511 ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C	584
<i>EPEC</i>	E2347	482
<i>eae</i>	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	
<i>bfp</i>	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT	300
<i>ETEC</i>	E5798	218
<i>lt</i>	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	
<i>stII</i>	E4683 AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C	129
<i>EIEC</i>	E35990	618
<i>virF</i>	AGC TCA GGC AAT GAA ACT TTG AC TGG GCT TGA TAT TCC GAT AAG TC	
<i>ipaH</i>	CTC GGC ACG TTT TAA TAG TCT GG GTG GAG AGC TGA AGT TTC TCT GC	933
<i>DAEC</i>	E66438	542
<i>daaE</i>	GAA CGT TGG TTA ATG TGG GGT AA TAT TCA CCG GTC GGT TAT CAG T	
<i>EAEC</i>	E60725	378
<i>aafII</i>	CAC AGG CAA CTG AAA TAA GTC TGG ATT CCC ATG ATG TCA AGC ACT TC	
<i>ETEC PCR</i> (Osek, 2001)		
<i>staI</i>	ICRV 214 (In-house control) TCTTTCCCTCTTTAGTCAG ACAGGCCGGATTACAACAAAG	166
<i>EAEC PCR</i> (Muller et al., 2007)		
<i>astA</i>	ICRV 181(In-house control) TGCCATCAACACAGTATATCCG ACGGCTTGTAGTCCTTCCAT	102
<i>EAEC multiplex PCR</i> (Cerna et al., 2003)		
<i>aap</i>	E60725 CTT GGG TAT CAG CCT GAA TG AAC CCA TTC GGT TAG AGC AC	310
<i>aggR</i>	CTA ATT GTA CAA TCG ATG TA AGA GTC CAT CTC TTT GAT AAG	457
<i>AA probe</i>	CTG GCG AAA GAC TGT ATC AT CAA TGT ATA GAA ATC CGC TGT T	629

Table 2

Distribution of virulence genes in DEC pathotypes

DEC Pathotypes	Cases (n = 394)	Controls (n = 198)	Pathogenicity index (only values >1 are shown)
Enteroggregative			
<i>E. coli</i>			
<i>aggR</i>	1	2	
<i>aap</i>	13	29	
<i>aap, aggR</i>	19	10	
<i>aap + aggR + AA</i>	24	6	2.01
<i>aafII</i>	3		
<i>astA</i>	74	55	
EPEC			
<i>bfp, eae</i>	24	5	2.38
<i>Eae</i>	15	11	
ETEC			
<i>Lt</i>	6	7	
<i>stII</i>	3	1	1.52
<i>stI</i>	4		
<i>lt, stII</i>	1		
<i>stII, stI</i>	1		
<i>lt, stI</i>	1		
EHEC			
<i>stx1</i>	4		
<i>stx1, eae</i>	2		
<i>stx2, eae</i>	2		
EIEC			
<i>virF, ipaH</i>	4		
DAEC			
<i>daae</i>	2		

Table 3

Association of different pathotypes of *E. coli* with clinical features of acute gastroenteritis^a

Pathotype	Diarrhea in days ^b (n = 375) ^c	P value	Max no. of diarrheal stools/day ^b (n = 370) ^c	P value	Vomiting ^d (n = 368) ^c	P value	Max no. of episodes of vomiting/day ^b (n = 360) ^c	P value	Fever ^d (n = 353)	P value
EPEC (n = 24)	2.5 (4)	0.988	5 (6)	0.291	17	0.651	3 (4)	0.662	14	0.689
AEPEC (n = 15)	3 (1)	0.96	7.5 (5)	0.764	14	0.206	4.5 (5)	0.222	8	1.00
EAEC (n = 56)	2 (2)	0.235	8 (7)	0.469	47	0.135	4 (4)	0.03	26	0.194
ETEC (n = 15)	2 (2)	0.148	5 (6)	0.115	13	0.322	3.5 (4)	0.175	8	1.00
EHEC (n = 8)	2 (2.5)	0.346	5 (5)	0.089	5	0.655	1 (4)	0.266	6	0.132
Coinfection (n = 15)	3 (2)	0.867	5 (8)	0.422	13	0.537	5 (7)	0.02	9	0.657

AEPEC = atypical EPEC; coinfection = coinfection with more than 1 putative enteropathogen.

^a Pathotypes infecting less than 5 children were excluded.^b Median (IQR).^c Data missing for some children.^d Number (%).