MOLECULAR DIAGNOSIS

Use of PCR to identify enteroaggregative *Escherichia coli* as an important cause of acute diarrhoea among children living in Calcutta, India

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The importance of enteroaggregative *Escherichia coli* (EAggEC) as a possible aetiological agent of acute diarrhoea among children in Calcutta, India, was investigated. Simultaneously the use of a previously described PCR diagnostic system was assessed for its ability to identify EAggEC infection. *E. coli* strains isolated during a 1-year case-control study from faecal samples of 388 children aged <5 years, with or without diarrhoea, were examined for EAggEC by HeLa cell adherence assay in parallel with a PCR assay with primers generated from an EAggEC DNA probe. A blind comparison was made between the two methods to determine their diagnostic potential. *E. coli* isolates that adhered to HeLa cells in an aggregative pattern were the sole isolates significantly more often in 254 cases (9%) than in 134 control (2%) children. Age stratification showed that EAggEC were isolated more frequently from children aged <36 months. The EAggEC isolates belonged to several O serogroups and showed multiple drug resistance. Both methods were positive for 26 samples, nine samples were positive by PCR alone and seven samples were positive by culture alone, thus indicating a 78% sensitivity and 97% specificity for the PCR assay. EAggEC is an important aetiological agent of acute diarrhoea among infants in and around Calcutta, and the PCR diagnostic system may be useful to identify such infection in epidemiological studies.

Introduction

Bacterial adherence to the intestinal mucosa is characteristic of *Escherichia coli* strains that cause diarrhoea in infants [1]. HEp-2 or HeLa cell in-vitro adherence assays are used to identify enteroadherent *E. coli* [2]. Three distinct patterns of adherence are described [3] – localised adherence, diffuse adherence and aggregative adherence. Enteroaggregative *E. coli* (EAggEC), which are characterised by typical man-nose-resistant stacked-brick-like adherence to cultured HeLa (or HEp-2) cell surfaces as well as on plastic or glass surfaces [2, 3], are of particular interest because of their unusual microbiological properties [4] and their epidemiological association with persistent diarrhoea in children in developing countries [5–7].

At present, the epidemiology of infection caused by EAggEC remains controversial. Although its associa-

tion with acute diarrhoea has been documented by some workers [8, 9], others have reported inconclusive results [10, 11]. EAggEC strains have also been isolated in outbreaks affecting young children and elderly patients [12–14].

The pathogenesis of EAggEC diarrhoea is also poorly understood. The presence of a large plasmid (60 MDa) confers the expression of the aggregative phenotype [4], which for certain strains is mediated by aggregative adherence fimbriae (AAFS) [15, 16]. EAggEC strains also secrete putative heat-stable and heat-labile enterotoxins [17, 18], but their role in diarrhoea has not yet been established [19]. More recently an autotransporter enterotoxin has been detected in isolates from a severe outbreak of diarrhoea [20].

Though EAggEC was originally identified by a HEp-2 cell adherence assay, the phenotypic characteristics are sometimes difficult to discern and subject to individual interpretation, as observed in a previous report [21]. Therefore, the development of genetic diagnostic tools will greatly facilitate the identification of such strains. A sensitive and specific DNA probe for EAggEC has

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been constructed from a 1-kb fragment of DNA from the 60-MDa plasmid of the prototype strain (17-2) isolated from a Chilean patient [22]. Based on the sequence of a fragment of the EAggEC probe, a PCR assay has been developed to identify EAggEC infection [23]. Some reports suggest that aggregative adherence fimbriae are not universally present in organisms that clearly have the aggregative phenotype [13, 24].

The present study was initiated to assess the importance of EAggEC as a possible aetiological agent of acute diarrhoea among children in Calcutta, India. The *E. coli* isolates obtained by coproculture were examined for mannose-resistant aggregative adherence to HeLa cells. Simultaneously the use of a previously described PCR diagnostic system [23] was assessed to characterise *E. coli* strains manifesting a true aggregative pattern of adherence.

Materials and methods

Study population

This hospital-based case-control study was done between May 1996 and April 1997. A total of 254 children aged <5 years, with acute diarrhoea, who were admitted to the Diarrhoea Treatment and Training Unit of the Dr B. C. Roy Memorial Hospital for Children, Calcutta, without any history of prior antibiotic therapy, were enrolled as cases in the study irrespective of type and duration of diarrhoea. Children (134) matched for age, sex, nutritional status and socio-economic status, who attended the paediatric clinic of that hospital without any history of diarrhoea or antibiotic therapy for the previous 2 weeks, were also included as controls. One control child was enrolled for every two diarrhoeic children and the seasonal distribution of controls and patients with diarrhoea was similar.

Acute diarrhoea was defined as an attack of sudden onset with passage of three or more liquid or semi-solid stools or one stool with altered consistency and blood within 24 h. Diarrhoeal episodes separated by ≥ 48 h were considered to be distinct episodes.

Microbiological methods

Stool samples were collected from the study population and were examined in the microbiology laboratory within 2 h of collection for the detection of enteropathogens. Stool samples were examined microscopically for trophozoites and cysts of *Entamoeba histolytica* and *Giardia lamblia*. Oocysts of *Cryptosporidium* spp. were also sought by the acid-fast Ziehl-Neelsen method. All samples were also tested for the presence of rotavirus antigen by PAGE. Identification of *Shigella*, *Salmonella*, *Aeromonas* and *Campylobacter* spp., *Vibrio cholerae* and *V. parahaemolyticus* isolates was performed by standard methods [25].

Identification of *E. coli* strains

Two lactose-fermenting and one (if any) non-lactose-fermenting colonies on a MacConkey Agar (Difco) plate were selected randomly from each patient and control and tentatively identified by biochemical tests as *E. coli*. Identified colonies were preserved on Dorset egg-yolk slants and tested blind by the following methods, independently.

Differentiation of pathogenic *E. coli* strains

A total of 803 *E. coli* isolates from 388 samples was tested by a PCR technique [26] with oligonucleotide primers to differentiate enterotoxigenic [27, 28], verotoxigenic [29] and enteroinvasive [30] *E. coli* (ETEC, VTEC and EIEC). Each strain could be identified by its distinct amplicon size. The specific D-mannose-resistant adherence patterns of localised, diffuse and enteroaggregative *E. coli* were detected by HeLa cell adherence assay as described previously [31]. Suitable positive and negative control strains were included in each study.

HeLa cell adherence assay

Briefly, 20 μ l of an overnight bacterial culture in Tryptic Soy Broth (Difco) were inoculated onto a monolayer of HeLa cells on glass coverslips in tissue culture plates, in the presence of methyl- α -D-mannoside 1%, and incubated at 37°C with CO₂ 10% v/v for 3 h. The petri dishes were washed three times, cells were fixed with methanol 70% v/v and stained with Giemsa 10% stain. Coverslips were mounted on glass slides and examined for adherence at $\times 1000$ magnification by light microscopy.

Additional EAggEC characterisation by PCR

All *E. coli* isolates were suspended in Luria Broth (Difco) and incubated at 100°C for 20 min. The boiled suspension was used as the source of DNA for PCR. The oligonucleotide primers PCVD 432/start (5' CTG GCG AAA GAC TGT ATC AT-3') and PCVD 432/stop (5' CAA TGT ATA GAA ATC CGC TGT T-3') were selected from the EaggEC probe [23]. The reaction mixture (50 μ l) consisted of boiled suspension 10 μ l, 50 mM KCl 10 mM MgCl₂, gelatin 0.01%, 0.25 mM each deoxynucleotide triphosphates, 50 pM of each primer and 2 units of Taq polymerase. The amplification was performed in a thermocycler (FTS 320, Corbett Research, Australia) for 30 cycles (94°C for 40 s, 53°C for 60 s, 72°C for 60 s) followed by a final extension at 72°C for 10 min. The identity of the amplified band at the 630-bp region was confirmed by Southern hybridisation with the specific probe for aggregative adherence [22]. Signals were visualised by chemiluminescence (ECL; Amersham, Life Science). Strain 17-2 was used as a positive control and reaction mixture without template DNA was used as reagent

control in each batch of tests. A comparison was made between the results obtained by the PCR method in relation to the results obtained by the HeLa cell adhesion method.

Serogrouping and antimicrobial susceptibility patterns of EAggEC strains

The enteroaggregative *E. coli* strains isolated from stool samples were further characterised for their O serogroup by slide agglutination with polyvalent and monovalent antisera (Denka, Seiken, Japan) against the following O antigens: O1, O6, O8, O15, O18, O20, O25, O26, O27, O28ac, O29, O44, O55, O63, O78, O85a, O111, O112ac, O114, O115, O119, O124, O125, O126, O127a, O128, O136, O142, O143, O144, O146, O148, O151, O152, O157, O158, O159, O164, O166, O167, O168 and O169. Serogroups were confirmed by test-tube agglutination by standard procedures. The strains showing autoagglutination were reported as rough strains.

Antimicrobial susceptibility testing was done by the disk diffusion method in accordance with National Committee for Clinical Laboratory Standards guidelines.

Statistical analysis

Fisher's two-tailed exact test and the χ^2 test were used for statistical analysis. Odds ratio (OR) with 95% confidence intervals (CI) were used as measures of association; $p < 0.05$ was considered significant.

Results

During the 1-year study period, 254 children <5 years of age admitted to the children's hospital had acute diarrhoea. The median age was 11 (min 1, max 60) months, and 104 (41%) patients were girls. Of the 254 cases, 180 (71%) had watery diarrhoea and 74 (29%) had mucoid diarrhoea with or without blood. Standard culture techniques detected bacterial, viral and parasitic enteropathogens in the stool of 180 (71%) diarrhoeic children and 16 (12%) healthy children. Multiple pathogens were detected in 43 (17%) cases and only 2 (1.5%) control children. The sole enteropathogens identified from cases were rotavirus (4.9%), *Shigella* spp. (4.1%) *Salmonella* spp. (2.3%), *V. cholerae* (1.9%), *Campylobacter* spp. (2.2%), *Aeromonas* spp. (2.4%), *Cryptosporidium* (1.9%), *Giardia lamblia* (1.7%), *Ent. histolytica* (0.8%).

Diarrhoea-producing *E. coli* constituted the most frequently identified organism, being the sole isolate in 81 (31.8%) cases and 11 (8%) controls ($p < 0.05$). Enteroadherent *E. coli* represented the most common category among enterovirulent *E. coli* and was identified in 59 (23.2%) cases and 11 (8.2%) controls. The

isolation rate of ETEC as sole agent was 8% (20 of 254) in diarrhoeic cases. Patients harbouring EIEC were very uncommon (2 of 254; 0.7%). VTEC were not identified from diarrhoeic cases. Control children did not have ETEC, EIEC or VTEC in their stools.

Identification and characterisation of EAggEC strains

EAggEC organisms in this study were identified primarily by phenotype, so all strains considered to be EAggEC were phenotypically positive, by definition, in the adherence assay. Table 1 shows that the isolation rate of EAggEC from cases (25 of 254; 9.8%) was significantly ($p < 0.05$) higher than that from controls (3 of 134; 2.2%). Overall, there was an association between the presence of EAggEC in stool and acute diarrhoea (OR, 4.8; 95% CI: 1.41, 25.1) in children.

However, age stratification showed the highest (4 of 26; 15.4%) isolation rate of EAggEC in infants aged <6 months (Table 2). Yet, because of the small sample size, the 95% CIs are broad, from +1% to -29%. In children >36 months, the presence of EAggEC in stool was not associated with diarrhoea.

Table 3 presents the clinical profiles of the patients infected solely with EAggEC. In four diarrhoeal children and one control child, EAggEC were isolated in association with other enteropathogens, e.g., rotavirus (two cases), *V. cholerae* (two cases), campylobacter (one control).

Table 4 shows a comparison of the results obtained by

Table 1. HeLa cell adhesion of enteroadherent *E. coli* isolated as sole pathogen

<i>E. coli</i> isolates with types of adhesion	Number (%) of infected children		p value
	diarrhoeal (254)	non-diarrhoeal (134)	
LAEC	16 (6.3)	1 (0.7)	0.006*
DAEC	18 (7.1)	7 (5.2)	0.48†
EAggEC	25 (9.8)	3 (2.2)	0.003*
Total	59 (23.2)	11 (8.2)	0.0001*

LAEC, localised adherent *E. coli*; DAEC, diffuse adherent *E. coli*; EAggEC, aggregative adherent *E. coli*.

*Fisher's exact test.

† χ^2 test.

Table 2. Age distribution of 388 children with or without diarrhoea from whom EAggEC were isolated

Age groups (months)	Number of infected children/number of children studied (%)	
	Cases	Control
<6	4/26 (15.4)	0/14 (0)
6-12	14/138 (10.1)	1/73 (1.4)
13-24	4/50 (8)	0/26 (0)
25-36	2/23 (8.7)	0/12 (0)
>36	1/17 (5.9)	2/9 (22.2)
Total	25/254 (9.8)	3/134 (2.2)

Table 3. Clinical profile of 25 children with diarrhoea solely infected with EAggEC

Characteristics	
Age (months): median (min, max)	10 (3.38)
Duration of diarrhoea in day before hospitalisation: median (min, max)	3 (1, 15)
	Number (%) of children
Male:female	15 (60):10 (40)
Stool character	
Watery	18 (72)
Mucoid	7 (28)
Bloody	0
Dehydration	
None	6 (24)
Moderate	9 (36)
Severe	10 (40)
Other features	
Vomiting	3 (12)
Fever (> 38.2°C)	4 (16)
Abdominal pain	6 (24)

Table 4. Results obtained by PCR for EAggEC in relation to results obtained by HeLa cell adherence assay

PCR result	HeLa cell adherence results		
	Positive*	Negative	Total
Positive	26	9	35
Negative	7	346	353
Total	33	355	388

Data are expressed as number of infected children.

*Positive for aggregative adherence.

PCR with those obtained by HeLa cell culture in the detection of EAggEC infection in children with or without diarrhoea. The PCR assay correctly detected EAggEC infection in 26 children, although 33 children showed the presence of EAggEC in their stools by HeLa cell adherence assay. Therefore, PCR failed to detect the infection in seven children from whom EAggEC were detected by HeLa cell culture. PCR was not positive for strains showing localised adherence. Nine samples showed positivity by PCR alone, of which *E. coli* isolates from two samples showed the diffuse type of adherence, and other strains did not show the typical stacked-brick appearance in the HeLa cell assay. Hence, the PCR assay was moderately sensitive (78%) and specific (97%) in this study. The hands-on time required in the laboratory to perform the PCR was only 2 h (SD 1.8) for testing 20 samples. Samples could be processed within 1 day after collection and overnight growth; the mean time required for the diagnosis of positive or negative samples by PCR assay, was 27 h (SD 5.6).

The results of analysing EAggEC isolates by HeLa cell assay, PCR assay and O serogroups are shown in Table 5. The O serogroups identified among the EAggEC isolates in this study were O44 (two strains), O128

Table 5. Analysis of the sole EAggEC isolates by HeLa cell culture, PCR and O serogrouping

EAggEC strains isolated	Number (%) of infected children	
	Cases (254)	Controls (134)
AA ⁺ , PCR ⁺	21 (8.2)	3 (2.2)
O serogroup	10	1
OUT	11	2
AA ⁺ , PCR ⁻	4 (1.5)	0
O serogroup	0	0
OUT	4	0
AA ⁻ , PCR ⁺	3 (1.2)	2 (1.5)
O serogroup	1	0
OUT	2	2

AA, aggregative adherence to HeLa cells; OUT, O serogroup untypable.

(three), O111 (three), O114 (one), O1 (one), O86 (one) and 17 strains were O untypable. Antimicrobial susceptibility patterns of the EAggEC isolates were uniformly (100%) resistant to ampicillin/amoxycillin and furazolidone; > 80% of strains were resistant to tetracycline and co-trimoxazole, 50% were resistant to nalidixic acid and 33% were resistant to chloramphenicol. However, almost all strains were uniformly (> 98%) sensitive to gentamicin, amikacin, norfloxacin, ciprofloxacin and cefotaxime; only one strain showed resistance to norfloxacin and ciprofloxacin.

The EAggEC strains isolated in this study did not possess enterotoxin genes (LT, ST or VT) or the enteroinvasive gene, as determined by PCR.

Discussion

The role of localised adherent *E. coli* as an important diarrhoea-causing agent in hospitalised infants in Calcutta, India was reported in a previous study [21], but EAggEC were not identified. The present study describes the association of EAggEC with acute diarrhoea in children (predominantly infants) in Calcutta; although the association decreased with age in children aged > 36 months. This observation was in accord with the observations of an earlier study [9], in which EAggEC was isolated significantly more frequently from patients with acute diarrhoea than from controls in the 0–2 months age group. Further studies are required to elucidate the risk factors among infants for developing diarrhoea due to EAggEC.

The clinical characteristics of diarrhoeic children infected solely with EAggEC indicated that acute watery diarrhoea (72%) was more frequent than mucoid diarrhoea (28%). None of the children had blood in the stool. Bhan *et al.* [5] reported gross blood in the stools of 12.2% of cases infected with EAggEC. The median age of infection of EAggEC was 10 months (min 3, max 38) and median duration of

diarrhoea was 3 days (min 1, max 15). Of 10 severely dehydrated children, nine were infants, three of whom experienced vomiting and required intravenous fluid for correction of dehydration. Fever and abdominal colic were detected in 16 and 24% of the infected children respectively. However, abdominal colic was observed more frequently among infected individuals in other studies [32].

The epidemiology of EAggEC has been difficult to study, because of difficulties in identifying the organism from stool cultures. Further studies are required to determine the source and transmission of infection for the development of effective control strategies to contain the infection. Recent reports of outbreaks [12–14] by EAggEC also support the need for epidemiological studies. Although EAggEC were originally identified by the HEp2 (or HeLa) adherence assay, this technique is cumbersome and inefficient if large numbers of samples are to be analysed, particularly in epidemiological studies. On the other hand O serogrouping is of little value in the identification of EAggEC, because in the present study only 39% of EAggEC isolates were O typable and they belonged to a number of O serogroups. Serotyping with specific 'H' antisera was not technically possible in this study. However, a similar observation of O untypable diarrhoea-associated EAggEC has been documented by other workers [9]. Hence, the use of molecular diagnostic systems in larger scale field studies needs to be evaluated.

The present study evaluated a PCR method described in an earlier report [23]. The data suggested 78% sensitivity and 97% specificity for this PCR method as compared with cell adhesion, which indicated considerable although not perfect correlation between the two methods. It was also observed that PCR was less time-consuming and simpler to perform than the cell culture technique. With proper standardisation, PCR may be performed directly on stool samples. However, to determine pathogenicity, it is necessary to identify the disease-causing strains.

Although it has been shown that these EAggEC strains lacked pathogenic factors such as the ability to express enterotoxins (LT, ST, VT) and invasiveness, studies are in progress to determine the possible virulence mechanisms, to explain the pathogenicity of EAggEC in children. The anomalies with respect to those strains detected by PCR alone or cell culture alone are to be studied in greater detail, to determine whether heterogeneity in PCR positivity is correlated with genetically heterogeneous types and virulence traits of EAggEC strains. Similar observations have been documented in previous studies [12, 24]. The reason why some EAggEC did not show positive results in the PCR assay is unknown [23], it is possible that there is sequence variation in the primer binding sites of those strains. Other diagnostic tools may be needed to

identify 20% (7 of 33) of the strains that were culture positive but PCR negative.

The antimicrobial susceptibility patterns of the EAggEC isolates showed multiple antibiotic resistance. Almost 60% of the isolated strains were resistant to five or more drugs and 22% were resistant to seven or more drugs. A previous study reported that EAggEC are fairly resistant to antibiotics and suggested that resistance may be linked to virulence [33]. Thus, a treatment schedule for children should consider the antibiotic resistance patterns of the local isolates. However, the role of chemotherapy in the treatment of EAggEC-associated diarrhoea is yet to be assessed.

Thus, the results suggest that EAggEC plays an important role in the aetiology of acute diarrhoea among infants in India and the PCR diagnostic system may be useful in epidemiological studies to identify EAggEC infection, although the definitive test for EAggEC remains the cell adherence assay.

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