JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 973–977
0095-1137/95/$04.00+0
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Vol. 33, No. 4

Controlled Study of *Escherichia coli* Diarrheal Infections in Bangladeshi Children

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Received 29 September 1994/Returned for modification 22 November 1994/Accepted 9 January 1995

Diarrheal diseases are highly prevalent in Bangladesh. However, the relative contribution of diarrheagenic *Escherichia coli* organisms—those that are enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive, enterohemorrhagic, enteroaggregative, and diffuse adherent—to diarrhea in Bangladeshi populations is not known. With DNA probes specific for these diarrheagenic *E. coli* strains, we analyzed fecal *E. coli* from 451 children up to 5 years of age with acute diarrhea seeking treatment at a Dhaka hospital and from 602 matched control children without diarrhea from July 1991 to May 1992. Enteroinvasive *E. coli* was not isolated from any children; enterohemorrhagic *E. coli* was not isolated from any diarrheal children but was isolated from five control children; enteroaggregative and diffuse adherent *E. coli* strains were isolated with similar frequencies from children with and without diarrhea, thereby showing no association with diarrhea; ETEC was significantly associated with diarrhea in the diarrheal children as a whole and especially in the age groups of 0 to 24 months and 37 to 48 months (further analysis suggests an association with diarrhea for the heat-stable toxin only and for both heat-labile- and heat-stable-toxin-producing ETEC only); and EPEC was significantly associated with diarrhea in the diarrhea group as a whole and particularly in infants up to 1 year of age. Further analysis suggested that EPEC strains of only the traditional serogroups were significantly associated with diarrhea. ETEC and EPEC infections peaked during warm months. Our data thus suggest that EPEC and ETEC are important causes of acute diarrhea in children in this setting.

Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries (31). Determination of the etiological agents of diarrhea is important in developing rational therapy and in implementing control measures. Continuing research has identified at least five categories of diarrheagenic *Escherichia coli*. They are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC), and diffuse adherent *E. coli* (DAEC) (27). It has become clear that there are important regional differences in the prevalence of different categories of diarrheagenic *E. coli* (4, 6, 8, 11). Moreover, studies in the past have been confined to defining the roles of limited categories of diarrheagenic *E. coli* by cumbersome phenotypic traits, and many such determinations rely on subjective tests (for example, tissue culture adherence assay for identification of EPEC, EAggEC, and DAEC) with a small number of subjects (6, 17, 20). The development of highly sensitive and specific probes for detection of all categories of diarrheagenic *E. coli* has dramatically altered the situation, making it comparatively easier to undertake comprehensive studies. Therefore, to define the association of various categories of *E. coli* with diarrhea, we carried out a controlled study using DNA probes on *E. coli* isolates from children up to 5 years of age.

MATERIALS AND METHODS

Patients. Children up to 5 years of age, seen between July 1991 and May 1992 at the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), located in Dhaka, were studied. They were part of the routine 4% surveillance sampling of all diarrheal patients seen at the Clinical Research and Service Centre (32). As part of the surveillance, relevant clinical information was routinely collected by means of a standard questionnaire. The information requested included age, sex, clinical picture (fever, vomiting, and dehydration status), type and duration of diarrhea, and history of antibiotic therapy prior to the clinic visit. Diarrhea was defined as the passage of three or more loose stools within the previous 24 h that conformed to the shape of the container. Dehydration was classified as none, mild, moderate, or severe according to standard criteria (13).

Controls. Control children without diarrhea matched for age, sex, and socioeconomic status were recruited from the nearby Dhaka Shishu (Children's) Hospital. These children attended the hospital for nondiarrheal illnesses during the same time as the children with diarrhea. They had not had diarrhea or antibiotic therapy in the preceding 2 weeks.

Microbiological studies. Fresh stool specimens obtained from patients were macroscopically examined for blood and mucus. Direct wet mounts of samples of unconcentrated feces were also examined for blood and pus cells as well as for *Entamoeba histolytica* and *Giardia lamblia*. The bacterial pathogens *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae* O1, as well as non-O1 vibrios, *Aeromonas* spp., and *Plesiomonas* spp. (collectively defined as other members of the family *Vibrionaceae*), were sought by standard methods (29, 34). Rotavirus was detected by an enzyme-linked immunosorbent assay (34). These pathogens were not sought in the stool specimens from control children; only *E. coli* isolates from these children were studied.

Diarrheagenic *E. coli*. Three lactose-fermenting colonies that morphologically resembled *E. coli* and any non-lactose-fermenting colonies were collected from MacConkey plates for each stool sample studied. These colonies were individually stored at −70°C in Trypticase soy broth (BBL, Becton Dickinson, Cockeysville, Md.) containing 20% glycerol. At the time of the study, they were subcultured once onto MacConkey agar and used to prepare filters for hybridization (see below). Only the non-lactose-fermenting colonies which were subsequently found to be *E. coli* by biochemical tests were hybridized.

DNA probes and hybridization analysis. The various DNA probes used for detection of diarrheagenic *E. coli* are shown in Table 1. DNA probes were prepared from recombinant plasmids containing the probe DNA fragments as inserts. Two probes corresponding to *E. coli* strains producing human and porcine forms of heat-stable toxins (ST) were employed because both forms of ST-producing *E. coli* are known to cause disease in humans. Plasmids were prepared, purified, and digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.), and the appropriate restriction fragments were purified as described previously (14). DNA fragments were labeled

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TABLE 1. DNA probes used for detection of diarrheagenic *E. coli*

Organism	Pathogenic factor detected[a]	Recombinant plasmid containing the probe	Probe obtained with restriction enzyme	Reference
ETEC	LT	pCVD403	1.3-kb *Bam*HI	14
	Human ST	pCVD402	216-bp *Eco*RI	14
	Porcine ST	pCVD404	157-bp *Pst*I	14
EPEC	EAF	pJPN16	1-kb *Bam*HI-*Sal*I	14
	AE factor	pCVD434	1-kb *Sal*I-*Kpn*I	23
EIEC	Invasion	pRM17	17-kb *Eco*RI	14
EHEC	SLT-I	pJN37-19	1.1-kb *Bam*HI	14
	SLT-II	pNN110-18	1,850-bp *Sma*I-*Pst*I	14
EAggEC	AA	pCVD432	1-kb *Eco*RI-*Pst*I	3
DAEC	DA	pSLM852	450-bp *Pst*I	14

[a] AE, attachment-effacement; AA, aggregative adherence; DA, diffuse adherence.

by random priming (14) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International plc, Aylesbury, United Kingdom), and a random primer labeling kit (Bethesda Research Laboratories). Colony blots were prepared on Hybond-N filters (Amersham International) which were then processed and hybridized under stringent conditions as described previously (12). The positive control *E. coli* strains used for hybridization reactions included EDL933 (for Shiga-like toxin I [SLT-I] and SLT-II), C600(933J) (for SLT-I), C600(933W) (for SLT-II), B170 (for EPEC adherence factor [EAF] and attachment-effacement factor), 08-AD (for diffuse adherence), EDL1284 (for invasion), E2539-C1 (for heat-labile toxin [LT]), 5203-70 (for human ST), TX1 (for porcine ST), and JPN-10 (for aggregative adherence). The negative control *E. coli* strains included C600, HB101, HB101(pBR322), and ATCC 25922. All these strains were obtained either from the Centers for Disease Control and Prevention, Atlanta, Ga., or from the Center for Vaccine Development, University of Maryland, Baltimore.

E. coli strains positive for hybridization by the EAF probe and/or the *E. coli* attachment-effacement gene A (*eaeA*) probe are referred to as EPEC in this report.

Serogrouping. Boiled cells of all probe-identified EPEC isolates were serogrouped by a slide agglutination test using commercial antisera to EPEC serogroups (Difco Laboratories). The antisera represented the following serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158.

Statistical analysis. The prevalence of diarrheagenic *E. coli* in patients and that in controls were compared by a two-tailed χ^2 test with Yates' correction and Fisher's exact test (1).

RESULTS

A total of 451 children with diarrhea and 602 matched control children without diarrhea were studied. The rates of isolation of pathogens from children with diarrhea are shown in Table 2. Diarrheagenic *E. coli* was isolated from 45.2% (204 of 451) of children with diarrhea compared with 26.6% (60 of 602) of children without diarrhea ($P = 0.0001$). In children with diarrhea, EPEC had the highest prevalence, followed by ETEC, EAggEC, and DAEC. EIEC was not detected in any children; EHEC was not detected in any children with diarrhea but was detected in five control children. Of children with diarrhea, approximately 23% (104 of 451) were infected with more than one of the potential diarrheal pathogens for which we tested, while 19.7% (89 of 451) were colonized with both a diarrheagenic *E. coli* strain and one of the other enteric pathogens. Four diarrheal children were colonized with two categories of diarrheagenic *E. coli* (one child with ETEC and EAggEC and three children with ETEC and DAEC), while no control children were colonized with more than one category of diarrheagenic *E. coli.*

The comparison of isolation rates of diarrheagenic *E. coli* in patients and controls is shown in Table 3. ETEC was significantly associated with disease in the diarrhea group as a whole and particularly in the age groups of 0 to 24 months and 37 to 48 months. Of the 54 patients positive for ETEC, 26 were also infected with another recognized pathogen. Even after this mixed-infection category was excluded, the rate of monoinfec-

TABLE 2. Isolation of pathogens from the stools of children with diarrhea[a]

Pathogen	No. of children (%) positive
ETEC	54 (12.0)
EPEC	70 (15.5)
EAggEC	43 (9.5)
DAEC	37 (8.2)
EIEC	0 (0.0)
EHEC	0 (0.0)
Salmonella spp.	5 (1.1)
Shigella spp.	46 (10.2)
V. cholerae O1	43 (9.5)
Other *Vibrionaceae*[b]	52 (11.5)
Entamoeba histolytica	5 (1.1)
Giardia lamblia	3 (0.67)
Rotavirus	76 (16.9)

[a] 451 selected surveillance children up to 5 years of age who attended the Clinical Research and Service Centre, ICDDR,B, from July 1991 to May 1992 were studied.

[b] Includes *V. cholerae* non-O1, *Aeromonas* spp., and *Plesiomonas* spp.

TABLE 3. Diarrheagenic *E. coli* isolated from children with diarrhea[a]

Organism[b]	Age group (mo)	No. (%) of infected children		*P* value
		Diarrheal	Control	
ETEC	0–12	24 (9.9)	7 (3.7)	0.012
	13–24	18 (14.9)	1 (0.9)	0.0001
	25–36	4 (10.0)	4 (3.7)	0.13
	37–48	7 (20.0)	1 (1.1)	0.0081
	49–60	1 (7.7)	3 (3.1)	0.402
	All	54 (12.0)	16 (2.7)	0.0001
EPEC	0–12	48 (19.8)	13 (6.8)	0.0001
	13–24	15 (12.3)	7 (5.9)	0.084
	25–36	5 (12.5)	6 (5.6)	0.15
	37–48	2 (5.7)	3 (3.1)	0.56
	49–60	0 (0.0)	4 (3.1)	0.45
	All	70 (15.3)	33 (5.3)	0.0001
EAggEC	0–12	30 (12.4)	22 (11.5)	0.76
	13–24	9 (7.4)	15 (12.7)	0.17
	25–36	3 (7.5)	14 (13.0)	0.35
	37–48	1 (2.9)	12 (13.6)	0.079
	49–60	0 (0.0)	4 (4.2)	0.45
	All	43 (9.5)	67 (11.1)	0.40
DAEC	0–12	18 (7.4)	9 (4.7)	0.23
	13–24	13 (10.7)	16 (13.6)	0.50
	25–36	2 (5.0)	7 (6.5)	0.74
	37–48	1 (2.9)	4 (4.6)	0.67
	49–60	3 (23.1)	8 (8.3)	0.10
	All	37 (8.2)	44 (7.3)	0.59

[a] 451 diarrheal children up to 5 years of age and 602 matched controls were studied during July 1991 to May 1992 in Bangladesh.

[b] EHEC was isolated from five control children but no diarrheal children; EIEC was not isolated from any children.

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TABLE 4. ETEC isolated from children with diarrhea[a]

ETEC type	No. (%) of infected children		*P* value
	Diarrheal	Nondiarrheal	
LT only	8 (1.8)	5 (0.8)	0.170
ST only	31 (6.9)	4 (0.7)	0.0001
Both LT and ST	15 (3.3)	7 (1.2)	0.015

[a] 451 diarrheal children up to 5 years of age and 602 matched controls were studied during July 1991 to May 1992 in Bangladesh.

tion with ETEC was significantly higher in children with diarrhea (28 of 451, 6.2%) than the total isolation rate in control children (16 of 602, 2.7%) ($P = 0.007$). Further comparison of LT and ST production is shown in Table 4. Significant association with diarrhea was seen with ETEC producing only ST and ETEC producing both LT and ST; ETEC producing only LT was not significantly associated with diarrhea.

EPEC was significantly associated with diarrhea overall and particularly in the first year of life (Table 3). We further analyzed EPEC on the basis of EAF and *eaeA* probe positivities. In children with diarrhea, all EPEC isolates were positive with both probes. However, EPEC from seven control children were EAF^- but $eaeA^+$. All the EAF^+ and/or $eaeA^+$ EPEC were subjected to serogrouping with commercial sera to the 12 traditional EPEC serogroups. Of the 70 children positive for EPEC, 40 harbored a traditional serogroup. The serogroups detected were O114 (18 children); O127 (10 children); O125 (3 children); O126, O119, and O142 (2 children each); O128 and O55 (1 child each); and both O125 and O126 (1 child). Of the 33 control children positive for EPEC, 7 harbored traditional serogroups. The serogroups detected were O127 (2 children); O125 (2 children); and O26, O55, and O142 (1 child each). The difference in the prevalence of traditional EPEC serogroups between patients and controls was statistically significant (40 of 451 versus 7 of 602, $P = 0.0001$). Serogrouping of isolates which were EAF^- but $eaeA^+$ from control children revealed that none of these isolates belonged to the traditional EPEC serogroups. The EPEC isolates which could not be recognized with traditional EPEC antisera obviously belonged to nontraditional serogroups. These EAF^+ and/or $eaeA^+$, nontraditional serogroups were not associated with diarrhea (30 of 451 patients positive versus 26 of 602 controls positive, $P = 0.095$).

The rate of isolation of EPEC decreased with increasing age in both patients and controls (χ^2 for the trend is 15.06, $P = 0.0001$). However, no obvious trend in the isolation rate was observed with other categories of diarrheagenic *E. coli.*

Thirty-three percent (23 of 70) of EPEC infections were associated with another recognized pathogen. Even after the mixed-infection category was excluded, the rate of monoinfection with EPEC was significantly higher in children with diarrhea (47 of 451, 10.4%) than the total isolation rate in control children (33 of 602, 5.5) ($P = 0.0001$). Further analyses of mixed infections for traditional and nontraditional EPEC serogroups were carried out. Ten of the 40 patients who harbored traditional EPEC serogroups had a mixed infection with a recognized pathogen, in contrast to 13 of 30 patients who harbored nontraditional EPEC serogroups ($P = 0.10$).

EAggEC and DAEC were isolated with similar frequencies from diarrhea patients and controls, thereby showing no association with diarrhea for these organisms.

The monthwise prevalence of ETEC and EPEC infections in diarrheal children is shown in Fig. 1. Peak ETEC infection prevalence was found in the dry, warm month of March and the wet, warm month of August; peak EPEC infection prevalence was found in the warm months of February and July and the cold month of December.

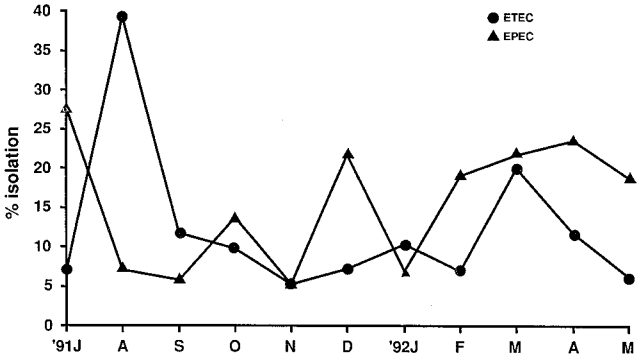

FIG. 1. Monthwise isolation rates of ETEC and EPEC from children with diarrhea. Months are listed by initial letter beginning with July 1991 ('91J).

Most of the patients had watery diarrhea, and they presented at the treatment center within a week of onset of diarrhea. Most of the patients had vomiting and various degrees of dehydration. More than half of the patients had a history of antibiotic therapy prior to presentation at the treatment center, and the majority had at least first-degree malnutrition. The mean age of the diarrheagenic-*E. coli*-infected patients was below 2 years.

DISCUSSION

As has been reported in many studies in developing countries (4, 11, 24), we found a strong association of ETEC with diarrhea in our children. No correlation between diarrhea and age was observed for the 25- to 36-month age group. The sample size for this age group might have been too small for us to detect a significant association. When further analysis based on the type of toxin produced was carried out, an association with diarrhea for ST^+ ETEC and LT^+ ST^+ ETEC was found. Thus, our findings are similar to the findings of several other studies (4, 11, 24).

EPEC continues to be an important cause of diarrhea in children up to 1 year of age in developing countries (26). EPEC can be identified by using a DNA probe based on the EAF plasmid (that mediates localized adherence through bundle-forming pilus [15]), a DNA probe based on *eaeA* (23), and the fluorescent actin staining (FAS) test, a phenotype-based tissue culture assay for attachment-effacement lesions (25). In the FAS test, the intimate attachment of the organism to the plasma membrane of the tissue culture cell triggers polymerization of actin filaments in the cytoplasm subjacent to the area of attachment of the organism. The polymerized actin is then detected by fluorescein isothiocyanate-conjugated phalloidin. In the present study, we employed all three tests, and a good correlation among them was achieved. Previous studies have shown good correlations between the *eaeA* probe assay and FAS test (23) and between the EAF probe assay and FAS test (30).

In children with diarrhea, all EAF^+ EPEC strains were also positive for *eaeA*, but EPEC strains isolated from several children in the control group were EAF^- but $eaeA^+$. Thus, it seems that EPEC isolates from control children were less pathogenic because of the inability of EAF^- strains to produce a bundle-forming pilus which mediates initial adherence of the organism to the intestinal mucosa (22).

Our study suggested that EPEC is associated with diarrhea

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in general and especially with the group up to 12 months of age. Further analysis based on serogrouping suggested that only EPEC strains of traditional serogroups were associated with diarrhea and no association with EAF^+ and/or $eaeA^+$ EPEC strains not belonging to the traditional serogroups was observed. These findings are in agreement with the results of several studies from other developing countries (9, 17–19, 28). This suggests that in addition to the *bfpA* (the gene that encodes subunits of the bundle-forming pilus) and *eae* genes, EPEC strains of traditional serogroups may possess an unknown virulence factor(s). Among the traditional EPEC serogroups identified in the present study, serogroups O114 and O127 predominated, and this is consistent with the findings of a previous study from Bangladesh (33). In other studies, children colonized with EPEC were rarely infected with other enteric pathogens (9, 17–19). A similar trend was apparent in the present study for the children infected with the traditional EPEC serogroups.

There are conflicting reports on the association of EAggEC and DAEC with acute and persistent diarrhea (2, 4, 6, 10, 16, 19). We found that these organisms are not associated with diarrhea. It is obvious that EAggEC and DAEC may represent heterogeneous groups of organisms, and certain subgroups within these categories may be pathogenic.

As found previously in Bangladesh (33), no child in the present study was infected with EIEC. Similarly, no child with diarrhea was infected with EHEC. The latter findings concur with the general finding in the countries of this region that the prevalence of EHEC infection is low (7). However, in a previous study in Bangladesh (33) in which oligonucleotide probes were used for detection of EHEC, EHEC was isolated from up to 9% of children with diarrhea. However, on subsequent testing, most of these isolates were found not to belong to EHEC.

The peak ETEC infection occurred in the wet warm month of August, as found in a previous study (5). However, peak rates of EPEC infection occurred mainly in the dry summer months of February to May. This trend agrees with the findings in other studies (18, 27).

There were no striking differences in the clinical presentations of children infected with various categories of *E. coli*. Also, the 247 children who did not harbor any probe-positive *E. coli* did not differ in their clinical presentation from those children who harbored probe-positive *E. coli*. More than half of the children in this study, when seen at the treatment center, had a history of prior antibiotic use. This may have affected the recovery of *E. coli* in some of these children. In a setting like ours, it is difficult to enroll children who have not had prior antibiotic therapy.

The prevalence of all six categories of diarrheagenic *E. coli* was studied previously in Bangladesh. However, these studies suffered from several deficiencies which included the following: (i) not all categories of *E. coli* were sought in a single study, which resulted in generation of data in a piecemeal fashion (2, 33); (ii) appropriate controls were not included, which made the interpretation of data difficult (21, 33); and (iii) some researchers relied on phenotype-based tissue culture assay for adherent *E. coli*, whose interpretation is prone to observer variation (2). The present study overcomes all these deficiencies and shows that only ETEC and EPEC are associated with diarrhea in this population of children.

ACKNOWLEDGMENTS

This study was funded by the United States Agency for International Development (USAID) under cooperative agreement DPE-5986-A-00-1009-00 with the ICDDR,B. The ICDDR,B is supported by the governments of Australia, Bangladesh, Belgium, Canada, China, Denmark, Germany, Japan, The Netherlands, Norway, Republic of Korea, Saudi Arabia, Sweden, Switzerland, the United Kingdom, and the United States. The ICDDR,B is also supported by the Arab Gulf Fund; Asian Development Bank; International Atomic Energy Centre; the United Nations International Children's Education Fund (UNICEF); the United Nations Development Programme (UNDP); the United Nations Population Fund (UNFPA); the World Health Organization (WHO); the Child Health Foundation; Ford Foundation; Population Council; Rockefeller Foundation; the Sasakawa Foundation; American Express Bank; Bayer AG; CARE; Family Health International; Helen Keller International; The Johns Hopkins University; Procter and Gamble; SANDOZ; Swiss Red Cross; the University of California, Davis; and others.

We thank Manzurul Haque for secretarial assistance.

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