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Isolation of Enterotoxigenic *Bacteroides fragilis* from Bangladeshi Children with Diarrhea: a Controlled Study

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We undertook a controlled study of children younger than 5 years in Bangladesh to determine whether enterotoxigenic *Bacteroides fragilis* (ETBF) was associated with diarrhea in this population. ETBF was isolated from 22 (6.1%) of 358 patients and 5 (1.2%) of 425 controls ($P = 0.0001$). In children younger than 1 year, however, low isolation rates (2 to 3%) were found in both patients and controls. In children older than 1 year, the rates were significantly higher in children with diarrhea (16 [9%] of 177) than in controls (2 [1%] of 264; $P = 0.00001$). When children with mixed infections with other known diarrheal pathogens were removed, the differences in children older than 1 year were still significant (7 [4%] of 177 versus 2 [1%] of 264; $P = 0.033$). The syndrome associated with ETBF was secretory in nature, with watery diarrhea, and of mild severity. These epidemiological and clinical findings are similar to those from a previous study of White Mountain Apaches in the United States and are the first to suggest that ETBF may also be an important diarrheal pathogen in other geographic areas and in the developing world where diarrhea is highly endemic.

Bacteroides fragilis bacteria, which are gram-negative, anaerobic rods and normal inhabitants of the large bowel of mammals, are of primary importance to humans when they cause opportunistic, extraintestinal infections. A subgroup of this species which produces an enterotoxin and which may be important as an etiologic agent of acute diarrheal illness in humans has now been identified. These enterotoxigenic *B. fragilis* (ETBF) organisms were first identified in diarrheal stools of young domestic animals in 1984 (6) and later, in 1987, were also identified in the stools of children and adults with acute and chronic diarrhea (7). The only published controlled study of ETBF as a possible cause of diarrhea in humans was done with the White Mountain Apache population in Whiteriver, Ariz.; it showed a strong association of ETBF with acute diarrhea in children older than 1 year (9). No controlled studies of ETBF infections in developing countries have yet been reported, although individual isolates have been found (4). This article describes the epidemiological and clinical association of these organisms with acute diarrhea in a developing country where diarrheal diseases are highly endemic.

MATERIALS AND METHODS

Patients. Children younger than 5 years admitted to the diarrhea hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh, with acute diarrhea between August 1991 and May 1992 were the patients for this study. They were part of the routine 4% surveillance sampling of all diarrhea patients seen at this hospital (12). As part of this surveillance, historical and clinical information is routinely collected by trained paramedical personnel using standard questionnaires and hospital records.

Controls were children younger than 5 years from similar socioeconomic environments, admitted within 2 weeks of the patients to the Dhaka Shishu (Children's) Hospital with non-diarrheal illnesses. Only those who had not taken any known antibiotics within 2 weeks of admission were enrolled in the study.

Microbiological assays. Whole stools (or rectal swabs) were examined microscopically and cultured for recognized enteropathogens according to the usual procedures in the International Centre for Diarrhoeal Disease Research, Bangladesh, laboratory (8, 12).

The following organisms were sought: rotavirus; *Vibrio cholerae* O1 and non-O1; and *Shigella*, *Salmonella*, *Aeromonas*, *Plesiomonas*, and *Providencia* spp. Diarrheagenic strains of *Escherichia coli* (enterotoxigenic, enteroadhesive, enterohemorrhagic, enteroinvasive, and enteroaggregative) were identified by DNA probes (2).

B. fragilis was cultured by direct plating on selective media. For most of the study, only PINN medium, which contains polymyxin B, irgasan, nalidixic acid, and novobiocin, was used (1). For the latter part of the study, BBE (*B. fragilis* bile-esculin agar) medium was also included (5); this medium contains gentamicin as the selective antibiotic. The plates were incubated under anaerobic conditions in a Gas Pack jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 2 days. Colonies were identified as *B. fragilis* if they had the characteristic internal mottled appearance when grown on PINN medium and were catalase positive and indole negative.

Enterotoxin production was assayed in HT29/C1 human colonic tissue culture cells (14) (kindly supplied by Cynthia Sears). Filtrates of *B. fragilis* colonies were made by growing them in brain heart infusion broth (Difco) for 48 h under anaerobic conditions and filtering the culture through a 0.22-μm-pore-size membrane filter. Filtrates were applied to semiconfluent monolayers of HT29/C1 cells, and morphological changes were read at 6 and 24 h (14).

As a confirmatory test for enterotoxin production, three *B. fragilis* strains that were positive in the tissue culture assay were tested in the modified RITARD (removable intestinal tie adult rabbit diarrhea) model as previously described (7). Each strain was inoculated (10^9 CFU) into four rabbits. One tissue culture-negative strain of *B. fragilis* was also inoculated at the same dose into four rabbits. The animals were observed for the presence of diarrhea over the next 7 days.

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TABLE 1. Isolation of ETBF

Age (mo)	No. with ETBF/total (%)	
	Patients [22/358[a,b] (6.1)]	Controls [5/425 (1.2)]
0–6	2/69 (2.9)	0/70
7–12	4/112 (3.6)	3/91 (3.2)
13–24	8/94[a,c] (8.5)	1/93 (1.0)
25–36	4/40[a,d] (10.0)	1/69 (1.0)
37–48	3/26[a,c] (11.5)	0/56
49–60	1/17 (6.0)	0/36

[a] Significantly different from the corresponding value for the controls.
[b] $P = 0.0001$.
[c] $P = 0.03$.
[d] $P = 0.06$.

Chi-square and Fisher's exact tests were used to compare differences between the groups.

RESULTS

There were 358 children with diarrhea and 425 matched controls studied. Historical information revealed that 150 (42%) of the cases had not received antibiotics before admission; 51 (14%) had received some antibiotics, with 30 (8% of total) having received metronidazole. The remainder (44%) had received some type of unspecified medication.

ETBF was isolated from 6.1% of children with diarrhea and 1.2% of children without diarrhea ($P = 0.0001$). The isolation rates according to age are shown in Table 1. It can be noted that isolation of ETBF was infrequent below 12 months of age in both patients and controls (2 to 3%) and the rates of isolation not significantly different between these groups. In all age groups over 12 months, however, ETBF was isolated at greater frequency (6 to 11.5%) and significantly more often in patients than in controls. The data, analyzed according to whether the children were younger or older than 1 year, showed that ETBF was isolated from 9% (16 of 177) of children older than 1 year with diarrhea and from only 0.8% (2 of 264) of controls ($P = 0.00001$). No differences were noted for children younger than 1 year: 3.3% (6 of 181) in subjects and 1.9% (3 of 161) in controls.

The data were further analyzed according to whether other enteropathogens had been isolated from the patients who harbored ETBF. From the 22 children with ETBF-associated diarrhea, 12 also had other pathogens isolated: rotavirus (two patients), *V. cholerae* (four patients), *Shigella flexneri* (one patient), *Shigella boydii* (one patient), *Salmonella* sp. (one patient), localized adherent *E. coli* (three patients), enterotoxigenic *E. coli* (one patient), and enteroaggregative *E. coli* (one patient). Among these 12 patients, 3 had more than one other enteropathogen isolated in addition to ETBF: one had *V. cholerae* and *S. boydii*, and two had *V. cholerae* and localized adherent *E. coli*. Among the five controls who harbored ETBF, one had an enteroaggregative *E. coli* and one had a localized adherent *E. coli*.

The data were again analyzed after the patients with multiple infections were excluded (Table 2). The number of children with diarrhea younger than 1 year from whom only ETBF was isolated remained small and not different from the corresponding number for the controls. The isolation rate of ETBF in children with diarrhea older than 1 year, however, remained significantly higher than that in children without diarrhea; 4.0% in patients compared with 0.8% in controls ($P = 0.33$ [Fisher's exact test, two-tailed]).

TABLE 2. Isolation of ETBF as the only enteropathogen

Age (yr)	No. with ETBF/total (%)	
	Patients	Controls
<1	3/181 (1.7)	3/161 (1.9)
1–5	7/177[a] (4.0)	2/264 (0.8)

[a] Significantly different from the corresponding value for the controls ($P = 0.033$; Fisher's exact test, two-tailed).

The 10 patients in whom ETBF could be identified as the only pathogen were analyzed according to their clinical signs and symptoms at the time of admission (Table 3). Seven of the children were older than 1 year, and nearly all were males. All children had watery diarrhea, but only three were mildly dehydrated on admission. Most had vomiting, none had fever, and two had abdominal pain. The illness was usually acute, with the median duration of illness before the child's coming to the treatment center being 3.5 days, although one child had a syndrome of persistent diarrhea (>15 days). The median number of stools per 24-h period was 8.

Using the modified RITARD model, 10 of the 12 rabbits (at least 3 of 4 rabbits inoculated with each strain of ETBF) developed diarrheal disease compatible with ETBF-induced diarrheal illness (7). Of the four rabbits inoculated with the tissue culture-negative strain of *B. fragilis*, one developed transient mild mucoid diarrhea on the third day following inoculation; the other three remained perfectly well.

A comparison of bacteriologic media for the isolation of *B. fragilis* was possible during this study. Toward the latter part of the study, BBE medium was added to the routine isolation procedures. Of 40 children with diarrhea, *B. fragilis* was isolated from 7 on BBE medium and from only 4 on PINN medium. From control children, 120 stools were processed by using both media; 31 (26%) were positive on BBE medium, and only 14 (12%) were positive on PINN medium. Of the total 160 stool samples for which both media were used, *B. fragilis* was isolated from 38 (24%) samples by using BBE medium and only 18 (11%) by using PINN medium ($P = 0.003$). BBE medium did not fail to grow any isolates that grew on PINN, but the PINN medium missed slightly over 50% of the *B. fragilis* organisms isolated on BBE.

TABLE 3. Clinical diarrheal syndrome symptoms in 10 children with only ETBF isolated from stool[a]

Parameter	Value
Age[b]	2, 6, 10, 18 (2), 24 (4), 36
Male/female	9/1
Watery diarrhea	10
Blood in stool	1
Leukocytes in stool[c]	1
Vomiting	6
Abdominal pain	2
Fever	0
Dehydration	3[d]
Duration[e,f]	3.5 (<1–>15)
No. of stools/day[f]	8 (3–15)

[a] Two of the patients had taken antibiotics prior to admission.
[b] In months. Numbers of children are indicated in parentheses.
[c] More than 20 leukocytes per high power field. Data for only eight children were available.
[d] The dehydration was mild. The other seven children showed no dehydration.
[e] Number of days.
[f] Median, with the range in parentheses.

DISCUSSION

This study has confirmed the previous one done by Sack et al. (9) in the Apache Reservation in the United States which showed that ETBF was epidemiologically associated with acute diarrheal disease in children older than 1 year. This study is the first, however, to be done in a developing country where acute diarrhea is highly endemic and the first to have included intensive microbiological studies. In the previous study the isolation of enteropathogens was limited, and therefore mixed infections could not be completely identified.

One of the confounding variables in such a study is the possible effect that antibiotic administration before admission might have on the stool culture results. As is found in most developing countries, some form of medication was taken before admission in 58% of our children with diarrhea, and 8% had taken metronidazole, to which *B. fragilis* is sensitive. This could have resulted in a falsely low isolation rate of ETBF in the children with diarrhea and blunted the differences between the groups, since none of the control children had taken antibiotics.

In neither of these two studies was ETBF isolated significantly more often in children younger than 1 year with diarrhea. This pattern is similar to that seen with other enteropathogens such as shigella and *V. cholerae*, which are isolated only infrequently in this young age group (3, 10). This observation may be partially explained by maternal antibodies which may be protective during this period or lack of exposure to the enteropathogens.

The large preponderance of males in this study (90%) is unexplained. The overall percentage of males in the study was 59%, which generally reflects the pattern of admission seen in Dhaka hospitals.

The clinical picture of the disease in children was one of acute watery diarrhea of a relatively mild nature, with no evidence of invasive disease (with one exception), i.e., no fever or blood in the stools. This is similar to the clinical description of the disease among Apache children (9). This clinical syndrome, acute secretory diarrhea, is consistent with the enterotoxigenic nature of the infecting organism. The intestinal secretory response is presumably coming from the large intestine, which is the normal habitat of *B. fragilis*. The enterotoxin of *B. fragilis* has now been purified (13), and its secretory mechanism of action is being investigated.

The isolation rates of *B. fragilis* were quite different in the two studies, although PINN medium was used as the primary selective medium in both. High isolation rates were found in Arizona (70% of all children [patients and controls] older than 1 year had *B. fragilis* [of any type] isolated from their stools), whereas low rates were found in Bangladesh (20%). Likewise, 20% of children with diarrheal disease older than 1 year had ETBF isolated from their stools in Arizona, but only 9% in Bangladesh. Part of this difference may be explained by the lower isolation rates of *B. fragilis* seen when PINN medium was used compared with BBE medium, an observation which suggests that the antimicrobial agents in the PINN medium were inhibitory to the *B. fragilis* strains resident in this part of the world. Additional laboratory studies (data not shown) have confirmed this observation.

Clearly, the isolation techniques are not yet optimal for isolation of all strains of *B. fragilis* in this population, and therefore our estimates of infection rates are undoubtedly low. In the controlled study design, however, this would not influence the comparative isolation rates. In future studies a selective medium, such as BBE, which is less inhibitory to these strains, needs to be used.

Another possible reason for the lower identification of ETBF in the present study is the assay system used. In the Arizona study the lamb ileal loop was the standard assay; in the present study a tissue culture assay, using unconcentrated culture filtrates, was used. Previous studies have shown these two assays to be comparable when a 20:1 concentration of culture filtrate is used. More recent studies have shown, however, that the enterotoxin can be detected by the tissue culture assay in unconcentrated culture filtrates, when Difco brain heart infusion broth is used (14). It is likely that more ETBF organisms would have been detected if the filtrates had been concentrated 20-fold.

Although *B. fragilis* is an anaerobic organism, it is aerotolerant, can survive for many months under aerobic conditions in the laboratory, and has been isolated from the effluent in sewerage plants (11). This suggests that it should be transmitted readily in an environment where fecal contamination is common.

This study strongly suggests that ETBF is an important etiologic agent in acute diarrhea in children older than 1 year in Bangladesh, as it is in Apache children in Arizona. Simpler techniques for its identification are clearly needed, so that additional studies can be carried out in other geographic areas. Such tests are currently being developed in our laboratory as well as those of others.

As newly recognized diarrheal pathogens continue to be described, it should be pointed out that the therapy of the acute illness does not usually depend on the identification of the many known enteropathogens. The major exception, of course, is the identification of shigella, in which the appropriate antibiotic is essential to successful therapy. Rehydration therapy (oral and intravenous) and continued feeding remain the cornerstone of therapy for the vast majority of acute diarrheal illnesses.

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