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Enterotoxigenic *Bacteroides Fragilis*: Epidemiologic Studies of its Role as a Human Diarrhoeal Pathogen

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ABSTRACT

Strains of *Bacteroides fragilis* which produce enterotoxin(s) (ETBF) have been associated with diarrhoeal diseases in young domestic animals and have also been isolated from humans with diarrhoea. We have determined epidemiologically that ETBF are significantly associated with diarrhoea in humans. We studied Apaches, primarily children, with diarrhoea attending an outpatient facility in Whiteriver, Arizona, from July 1986 through July, 1988. Stool cultures for isolation of ETBF and other diarrhoeal pathogens were taken from these persons as well as from age and time - matched control persons who did not have diarrhoea. ETBF were isolated significantly more often from persons with diarrhoea (12%) than from controls (6%), p = 0.03. Isolation was highest (20 - 24% of stool cultures positive) during the second and third years of life. The diarrhoeal syndrome associated with ETBF was non - specific, and most characteristic of a secretory, rather than inflammatory, type of diarrhoea. ETBF are significantly associated with acute diarrhoea in Apache children, and may be an important newly described cause of diarrhoea in humans.

Key words: *Bacteroides fragilis*; Diarrhoea, Acute; Diarrhoea, Infantile; Epidemiology.

INTRODUCTION

During the past eight years, strains of *Bacteroides fragilis* which produce enterotoxin(s) have been shown to be associated with acute diarrhoeal diseases in young animals (lambs, calves, pigs, and foals) (1 - 5). Enterotoxin production by *B. fragilis* was identified using the lamb ileal loop test (1); both live organisms and concentrated, bacteria - free culture filtrates caused fluid accumulation in the lamb ileum. These enterotoxigenic *B. fragilis* (ETBF) were subsequently found to produce severe diarrhoeal disease in several intact animals including gnotobiotic piglets (6), adult rabbits with ligated ceca (7) and, three - day - old (8) and 2 - weeks old rabbits (9). The characteristic pathologic lesions found during diarrhoea were primarily located in the colon and caecum in all these animals.

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Preliminary studies also showed ETBF excretion in faeces in some humans with acute and chronic diarrhoeal diseases (7). The present studies were done to assess the significance of ETBF in a human population, in persons with and without acute diarrhoeal disease. The results indicate that this organism is associated with acute diarrhoeal illness, and may be an important human diarrhoeal pathogen.

MATERIALS AND METHODS

Study Population. The study was carried out among the White Mountain Apache population living in Whiteriver, Arizona from July 1986 until July 1988. Persons of all ages who presented to the clinics at the Indian Health Service Hospital in Whiteriver with diarrhoeal disease were eligible for the study. No exclusion criteria were used. For each patient with diarrhoea, we selected one control patient who had not had diarrhoea within the past

two weeks, but had presented to the clinics with an unrelated illness. Each control patient was matched for age (age 0–6 months, within 1 month; age 6–24 months, within 3 months; 2–5 years, within 6 months, and above 5 years, also above 5 years) and for time of stool collection, within the two weeks following selection of the patient.

The study was approved by Committees on Human Volunteers of the Johns Hopkins University School of Hygiene and Public Health, the Indian Health Service and the Health Board of the White Mountain Apache Tribe.

Stool Cultures. Stool specimens from the patients and controls were cultured for *B. fragilis* by obtaining rectal swabs in modified Stuart's transport medium (Culturette II; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Missouri). The swabs were sent by courier mail and received within fourteen days of collection in our laboratory in Montana, where primary isolations were made. Each specimen was streaked for colony isolation onto PINN agar (2). Plates were incubated anaerobically (GasPak Anaerobe Systems: BBL Microbiology Systems, Cockeysville, MD.) for 48 hr at 37°C. From each PINN plate four colonies which had the characteristic internal mottled appearance of *B. fragilis* were picked and grown individually for an additional 48 hr under anaerobic conditions on tryptose blood agar base (TBA) (Difco Laboratories) supplemented with 5% defibrinated bovine blood, to check for purity. Isolates were identified as *B. fragilis* if they were catalase positive, indole negative, and if they did not ferment rhamnose, trehalose, or mannitol (10). Isolates were stored anaerobically for up to 1–2 months at room temperature on TBA slants before enterotoxin testing, and were held at −70°C for long-term storage.

Stool specimens from the patients with diarrhoea, but not from the control patients, were also cultured for *Shigella, Salmonella, Campylobacter*, and *Yersinia* according to standard methods in the Whiteriver Indian Health Service Hospital Clinical Laboratory (11). Identification of diarrhoeagenic *Escherichia coli* and rotavirus was not done.

Enterotoxin Assays. Enterotoxin activity was assayed by growing the isolates of *B. fragilis* anaerobically in brain heart infusion broth (BHIB; Difco Laboratories) for 24–48 hours at 37°C. One ml of whole bacterial culture from each isolate grown was tested for enterotoxin activity by assaying for fluid accumulation in the lamb ileal loop test (1). Approximately 40 cultures could be assayed in each lamb. A culture was considered enterotoxigenic if it resulted in the accumulation of more than 0.3 ml of fluid per cm of intestine with no measurable fluid in the adjacent loops, in at least two lambs. Most positive loops, however, had 1.0–1.5 ml fluid per cm of intestine. Negative loops were devoid of any measurable amount of fluid.

In most cases, four isolates of *B. fragilis* were tested from each stool specimen. In some stool specimens harboring ETBF, fewer than four isolates were tested when the initial isolate tested was found to be enterotoxigenic.

Restriction Enzyme Analysis. Selected isolates of ETBF were characterized by restriction enzyme analysis (REA). The DNA relatedness of these strains was assessed by comparing the electrophoretic pattern of a restriction enzyme (CfoI) digest of total DNA from each isolate, as described previously (8). Analysis was done using a commercially available assay kit (Globex Biotechnologies Inc., Toronto, Ontario, Canada). Isolates with identical electrophoretic profiles were considered to be the same strain.

Clinical Parameters of Disease. At the conclusion of the laboratory phase of the study, the clinical records of those patients harboring ETBF were examined for standard clinical parameters of diarrhoea. A matched group of children with diarrhoea from the study who had non-enterotoxigenic *B. fragilis* isolated from their stools were chosen for comparison of their clinical parameters. The matching criteria used were: (a) time, within 2 weeks of the case, (b) age, 0–12 months, within 2 months; 12–36 months, within 6 months; 3–5 years, within 2 years; above 5 years, also above 5 years.

Statistical Analysis. Chi-square and Fishers' exact tests were used on nominal data; a two tailed (Student's *t* test) was used to compare the interval data.

RESULTS

Three hundred and four persons with diarrhoea and 202 matched controls had stool samples taken for culture of *B. fragilis*. It was not possible, as indicated by the smaller number of controls, to match every case with a suitable control. Furthermore, when the study first began, some stool samples took longer than 14 days to arrive in the laboratory for culturing of *B. fragilis*; since recovery of *B. fragilis* from the swabs was found early in the study to be markedly reduced after 14 days (data not presented) these samples were excluded from the analysis (29 persons with diarrhoea, and 27 controls.) In addition, there were 5 stool swabs that had been collected incorrectly and consequently were excluded. The final analysis included, therefore, 275 persons with diarrhoea and 185 controls.

ETBF were isolated from 12% (33/275) of persons with diarrhoea as compared to 6% (11/185) of controls, a statistically significant difference ($p = 0.03$; Relative Risk 2.16, confidence interval 1.06–4.39). The isolation of ETBF, however, was

distinctly correlated with age, as shown in Figure 1. Children with diarrhoea under the age of one year had low isolation rates (8/147;5.4%) which did not differ from the controls. Children between the ages of 13 – 36 months had the highest isolation rates (20 – 24%) and the greatest differences from controls (8 – 9%). Although the differences between cases and controls in each of these two age categories (13 – 24 months and 25 – 36 months) were not significantly different when compared separately, when they were combined the differences were statistically significant (p = 0.03). For the age category above 3 years, there were only small differences that were not significant. When all persons above the age of one year were compared, however, the differences remained significant (p = 0.05).

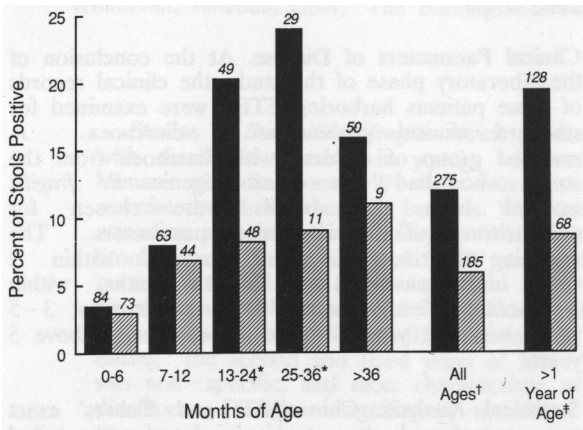

Figure 1. Isolation of enterotoxigenic *Bacteroides fragilis* from stools of persons with and without diarrhoea, grouped according to age. Solid bars indicate the patients with diarrhoea; bars with diagonal lines indicate the controls. Numbers above bars indicate the number of persons whose stool samples were cultured

*p = 0.03, when these two age groups are combined. +p = 0.05

As shown in Figure 2, the isolation rate for all *B. fragilis* (including non – enterotoxigenic and enterotoxigenic strains) increased with age until age one year, when it was about 70%; it remained constant at that level in all the older age groups. There were no differences in overall isolation rate for *B. fragilis* between persons with diarrhoea and controls, except for the 7 – 12 month old children, in whom the rate among controls was significantly greater than from persons with diarrhoea (p = 0.01).

The seasonal distribution of ETBF isolations was also examined. When all ETBF isolations in the warm months were compared with those in the cold months, there were no statistically significant differences in the percentage of cases harboring ETBF: April – September, 28/203;13.7%, vs October – March, 5/72; 7.0%, p = 0.20. In the control patients the rates were also similar: 8/120; 6.6% for the warm months and 3/65; 4.6% for the cool season.

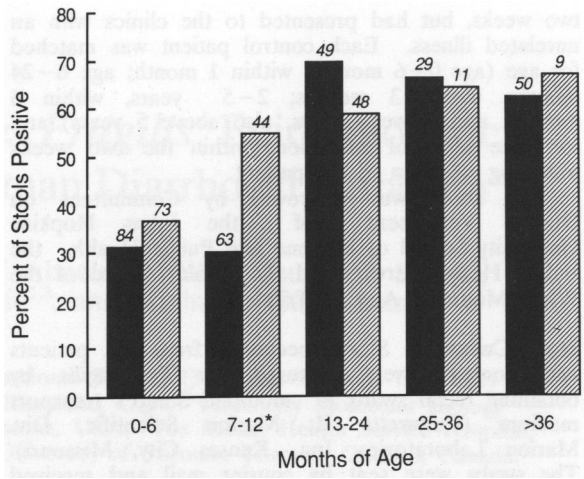

Figure 2. Isolation of all *Bacteroides fragilis* from stools of persons with and without diarrhoea, grouped according to age. Solid bars indicate the patients with diarrhoea; bars with diagonal lines indicate the controls. Numbers above the bars indicate the number of persons whose stool samples were cultured.

*p = 0.01

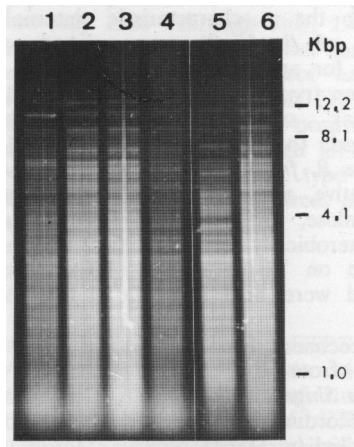

Figure 3. Agarose (0.7%) gel electrophoresis of total cellular DNA from enterotoxigenic *Bacteroides fragilis* isolated from stool of humans with diarrhoea. This reference gel demonstrates three of the major restriction enzyme analysis (REA) profiles. The DNA was digested with the restriction endonuclease Cfo I. Lanes 2, 3, 4, and 6 represent the Cfo I profile for the major REA Family 1; Lane 1 represents REA FAamily 1 – 2; and Lane 5 represents the profile of Family 2. Reference marker DNA is given in Kilobase pairs (Kpb).

In persons harboring ETBF, most of the *B. fragilis* isolates tested were enterotoxigenic, indicating that they made up the predominant part of the *B. fragilis* flora. From the 44 persons harboring ETBF, (33 with diarrhoea and 11 controls) 109 of the 130 isolates of *B. fragilis* tested (84%) were enterotoxigenic. There were no differences between the case and controls, however, as to the

percentage of *B. fragilis* isolates that were enterotoxigenic.

We examined whether the period of time within the 14 days of transit in holding media was a significant variable for isolation. The culture results were divided according to whether the stool specimens were cultured within 0–7 days or 8–14 days after stool collection. A significantly decreased isolation rate in older specimens for all *B. fragilis* (including ETBF) was found in persons with diarrhoea and in controls (155/289 (54%) for 0–7 days vs 71/171 (42%) for 8–14 days, $p = 0.05$).

There were three clusters of ETBF-positive diarrhoeal cases involving 16 persons that occurred during time periods of 1 to 7 days. The REA patterns of ETBF isolates from these clusters, summarized in Table I and Figure 3, indicate that a) in both instances, isolates from family members had the same pattern, and b) REA family 1 (or its variants) was the predominant strain (8/12 strains) involved. There were no clusters of ETBF found among the control patients.

Table I. Clusters of Enterotoxigenic *Bacteroides Fragilis* – Associated Diarrhoea

Cluster	Sample interval (days)	Restriction Enzyme Analysis (REA) Families*					
		1	1–1	1–2	2	2–1	3
1	Aug 3–7, 1986 (5)	2	–	1	–	–	–
2	Sept 1–7, 1986 (7)	3**	1	–	–	1	1
3	July 7, 1988 (1)	1	–	–	2§	–	–

* Only 12 of the 16 isolates for ETBF were characterised; REA patterns of the major families are shown in Figure 3.
** Two are brothers.
§ Two are sisters.

There were three individuals from whom ETBF were isolated on two separate occasions; in one person, both isolates were from normal stool specimens obtained 16 months apart; the REA showed them to be identical strains (REA Family 1). The other two persons had specimens taken within three months of each other; in each person, one was a diarrhoeal stool sample and the other, a normal stool sample. In one of these two persons, the strains were also identical by REA patterns (REA Family 2)

Stools were also cultured for a limited number of enteric pathogens from 175 of the 275 persons with diarrhoea. *Shigella* was the most common enteropathogen isolated (n = 19, 10.9%); 14 isolates were *S. flexneri*, 4 were *S. boydii*, and 1 *S. sonnei*. Other enteric pathogens included *Campylobacter jejuni* (n = 2) and *Salmonella* (n = 1). No enteric pathogens were isolated in 153 (87%) patients. From only one patient who harboured ETBF was another enteropathogen (*S. boydii*) isolated.

The results of the clinical record review of the 33 patients with diarrhoea from whom ETBF were isolated and the 33 matched patients with diarrhoea from whom non-toxigenic *B. fragilis* were isolated are summarised in Table II. The illness in persons harbouring ETBF was of about 3 days duration at the time of presentation, and was characterized by watery diarrhoea. The only significant differences between the two groups were in the lower occurrence of vomiting in the ETBF group, and the greater isolation rate of other enteric pathogens in the control group. There were no indications that the clinical illnesses associated with ETBF were particularly of an inflammatory nature, i.e. with fever or blood in the stools; the same clinical spectrum of illness was seen in the control group, 29% of which had invasive organisms isolated, predominantly *Shigella*.

Table II. Clinical characteristics of matched diarrhoeal patients from whom enterotoxigenic *Bacteroides fragilis* (ETBF) or non-enterotoxigenic *Bacteroides fragilis* were isolated

	ETBF isolated (n = 33)	Non-ETBF isolated (n = 33)
Age (years; mean ± SD)	3.2* ± 1.1	3.6 ± 1.4
median (years)	1.8	1.7
range (years)	0.1 – 37	0.3 – 42
Duration of illness before visit (days)	2.9 ± 2.7 (n = 28)	2.2 ± 2.2 (n = 24)
range	(1 – 14)	(1 – 10)
No. stools, last 24 hrs. before visit	6.5 ± 4.0 (n = 11)	4.8 ± 1.7 (n = 16)
range	(3 – 16)	(2 – 9)
No. with blood in stool	3 (9%)	4 (12%)
No. with fever	19 (58%)	21 (64%)
No. with vomiting**	7 (21%)	19 (58%)
No. with signs of upper respiratory infection	7 (21%)	11 (33%)
No. with clinical signs of dehydration	6 (18%)	9 (27%)
No. hospitalized	5 (15%)	9 (27%)
No. with enteric pathogens isolated π	1/23§ (4%)	7/24# (29%)

* mean ± S.D.
** p = 0.005
π p = 0.048, Fisher's exact Test
§ *Shigella boydii*
# 5 *Shigella flexneri*, 1 *Shigella boydii*, 1 *Campylobacter jejuni*

DISCUSSION

This study is the first attempt at determining whether ETBF are associated with diarrhoea in humans, and only limited supportive microbiological

diagnostic studies were possible. Nevertheless, although not providing final proof of their role as a diarrhoea pathogen, these results strongly indicate that the isolation of ETBF is significantly associated with acute diarrhoeal disease in children between the ages of 13–36 months in this population. Children 12 months of age or younger have relatively low isolation rates, similar to the controls. Similar age-related patterns of infection have been noted for *Shigella* (12) and *Vibrio cholerae* (13). The numbers of persons studied over the age of 3 years were relatively few, and no definitive statements can be made about these age groups.

It should be pointed out that this type of study is relatively insensitive in documenting pathogenicity of diarrhoeagenic organisms. There are several known diarrhoeal agents in which it is frequently not possible to establish differences between isolation rates in persons with diarrhoea and in controls. Included in this list are enterotoxigenic *E. coli* and *C. jejuni* (14).

There were no associations observed between ETBF and other enteropathogens isolated, although these studies were limited, and the study did not include rotavirus or enterotoxigenic *E. coli*, which are known to be important pathogens in this population (11). Even without these data, however, the association of ETBF with diarrhoea is striking. It is possible that this association could also have been with another known diarrhoeagenic agent, not detected here, but this would not have negated the association of ETBF with diarrhoea.

One of the limitations to the recovery of *B. fragilis* in this study was the time period between obtaining the samples and the plating. The recovery was higher when the transit time was less than one week. Since samples from both cases and controls were handled identically, however, this would not have influenced the differential isolation rates between the groups.

Since identical ETBF strains, as defined by REA analysis, were isolated during three outbreaks in several persons, and within families, this suggests that some of the transmission may have occurred from a common source. Unfortunately, it was not possible in the course of this study to identify possible methods of spread of the organism. Although *B. fragilis* is an obligate anaerobe, it survives in air for long periods of time, and therefore transmission by the usual fecal-oral routes should be readily accomplished. Furthermore, ETBF comprised about 9% of the total *B. fragilis* isolated in municipal sewage influent, (15), again demonstrating that the organisms are present, and can survive in the environment.

The studies of restriction enzyme analysis also make it possible to conclude that in some persons, these organisms are carried for many months without causing disease. The same ETBF, as indicated by REA, was isolated from the same person on two occasions, once during an asymptomatic period, and again two months later during an epsiode of diarrhoea. This pattern is somewhat similar to that of *Clostridium difficile*, another large bowel anaerobic organism that may be either a part of the normal flora or a causative agent of diarrhoea, through the production of enterotoxins (16).

The clinical data suggest that ETBF are not associated with a typical inflammatory type of diarrhoea. In fact, the clinical illness could not be differentiated from those patients matched for harboring non-enterotoxigenic *B. fragilis*, but who did have a significantly higher percentage of invasive organisms isolated. The marked inflammatory lesions seen in the adult rabbit with ligated caecum (7) had suggested that the clinical illness in humans might have included blood in the stool and fever, analogous to classical shigellosis. This clearly was not the case, and suggests that a more non-specific secretory watery diarrhoea syndrome is seen, as noted in the young rabbit (8,9) and piglets (6). We were not able to study persons with prolonged diarrhoea as part of this study, since these patients did not present to the treatment facility. This clinical syndrome requires additional investigation.

Before large scale studies can be done with this organism, clearly, a simpler enterotoxin assay than the lamb ileal loop is required. We are developing a tissue culture assay, using colonic epithelial cell lines, that will simplify the identification of ETBF. Preliminary studies indicate that culture filtrates of ETBF effect morphologic changes in the colonic cell line HT29/C1 clone (17). Such an assay will allow studies of toxin purification and immunologic properties to proceed.

In summary, ETBF, which are diarrhoeagenic in a number of animal models, and are isolated frequently from young animals with severe diarrhoeal disease, are now shown to be associated with diarrhoea in young children. Additional studies in other geographic areas in which complete aetiological studies can be done and where prospective clinical data can be obtained will be needed to describe further its role as a possible diarrhoea pathogen. At some time in the future, volunteers will need to be challenged with these organisms to clearly establish their pathogenicity.

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