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Isolation of Enterotoxigenic *Bacteroides fragilis* from Humans with Diarrhea†

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Enterotoxigenic *Bacteroides fragilis* was isolated from stool specimens of 8 of 44 diarrheic individuals (ages, 4 months to 69 years). The individuals had watery diarrhea and intestinal cramping; and infants had hyperthermia, vomiting, and blood in the stools. No recognized enteric pathogens were detected in seven of the eight diarrheic individuals positive for enterotoxigenic *B. fragilis*. The bacterium produced an enterotoxin detectable in concentrated broth that supported bacterial growth. Fifteen adult rabbits with ligated ceca developed fatal enteric disease following intraileal injection with 5×10^9 CFU of enterotoxigenic *B. fragilis*. Conversely, eight control rabbits injected with nonenterotoxigenic *B. fragilis* remained clinically normal. As few as 5×10^3 CFU of enterotoxigenic *B. fragilis* caused fatal enteric disease in the rabbit model. Disease in rabbits was characterized by mucoid, often hemorrhagic, diarrhea. The bacterium colonized the caudal small intestine and the colon of the rabbits and caused moderate to severe necrotizing colitis. Enterotoxigenic *B. fragilis* is widespread in the intestinal tract of diarrheic humans and is enteropathogenic in adult rabbits with ligated ceca. Its possible role in the enteric disease complex merits further study.

Bacteroides fragilis is a gram-negative, non-spore-forming, obligately anaerobic bacterium found in high numbers in the intestinal tracts of humans and animals. The bacterium is recognized as an important cause of abscesses and other extraintestinal infections in various tissues in humans but is not recognized as an enteric pathogen in humans.

While most intestinal isolates of *B. fragilis* are nonenterotoxigenic, it has been shown (8) that some animal isolates elaborated an enterotoxin into the medium during growth in vitro. Enterotoxin activity was detected in concentrated (but not in unconcentrated) broth by using the calf or lamb ligated ileal loop (LIL) test. Enterotoxigenic *B. fragilis* has been isolated from the feces of diarrheic lambs (5), calves (8), pigs (7), and foals (L. L. Myers, D. S. Shoop, and T. D. Byars, Am. J. Vet. Res., in press). Severe enteric disease occurred in adult rabbits with ligated ceca following intraileal injection of 5×10^9 CFU of one of three different porcine isolates of enterotoxigenic *B. fragilis* (7). In the present study, it is shown that enterotoxigenic *B. fragilis* can also be isolated from diarrheic humans and that human isolates of enterotoxigenic *B. fragilis* are virulent in the adult rabbit model.

MATERIALS AND METHODS

Isolation of *B. fragilis*. Seventy-six stool specimens from 44 diarrheic individuals were obtained by using rectal swabs (Culturette II; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.). Of the 44 individuals studied, 34 were infants, (age, between 2 and 14 months) from the Navajo Area Indian Health Service, Tuba City, Ariz. These individuals were examined for *Salmonella*, *Shigella*, *Aeromonas*, and *Yersinia* species; *Campylobacter jejuni*; enterotoxigenic *Escherichia coli*; and rotavirus, as described previously (9). The other 10 diarrheic individuals were patients at a private medical practice (Medical Associates) in Bozeman, Mont. These individuals were studied because they had diarrhea of unknown cause. No *Salmonella*, *Shigella*, *Campylobacter*, or *Giardia* species were detected prior to the attempted isolation of enterotoxigenic *B. fragilis* from these 10 individuals.

Stool specimens on swabs were streaked onto a solid, selective medium (1) composed of tryptose blood agar (TBA; Difco Laboratories, Detroit, Mich.) plus polymyxin B, triclosan (Irgasan), novobiocin, and nalidixic acid. Plates were incubated anaerobically (GasPak anaerobe system; BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C. Bacteria from four colonies with the characteristic internal mottled appearance of *B. fragilis* were grown individually and presumptively identified as *B. fragilis* if they were catalase positive and indole negative and if they did not ferment rhamnose, trehalose, or mannitol (2, 3). Confirmatory testing of five isolates was done at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. Isolates of *B. fragilis* were stored aerobically for up to 2 months at room temperature on TBA slants. For long-term storage, the isolates were transferred to glass culture tubes (6 by 50 mm) containing defibrinated bovine blood. The tubes were heat sealed and held at −80°C.

Enterotoxin studies. Isolates of *B. fragilis* were grown on TBA, washed off the plates (100 by 15 mm), and tested for enterotoxin activity (fluid accumulation) in the lamb LIL test (4, 8). Isolates were considered enterotoxigenic if they caused loops to be at least one-half filled with fluid (≥0.3 ml of fluid per cm of intestinal wall) while adjacent loops were devoid of measurable fluid.

To further study the enterotoxin, two isolates (2-078382-3, 20793-3) of enterotoxigenic *B. fragilis* and two isolates of nonenterotoxigenic *B. fragilis* were each grown in 800 ml of

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TABLE 1. Cases of enterotoxigenic *B. fragilis*-associated enteric disease

Patient (age; *B. fragilis* isolate)	Case description
Male (69 yr; 102-4-2)	Diarrhea for 4 wk
Female (43 yr; 20656-2-1)	Intermittent diarrhea of at least 3-yr duration (still present in May 1987); mild intestinal cramping; enterotoxigenic *B. fragilis* was detected on three occasions (January, April, and June 1986) and was not detected in May 1987
Male (3 yr; 20839-2)	Diarrhea for 10 days; intestinal cramping; temp, 37.8°C
Female (23 mo; 20793-3)	Diarrhea for 4 wk; occasional hemorrhagic mucus in stools
Three males, one female (5–8 mo; 078044-1, 2-078382-3, 078320-3, 079298-3)	Diarrhea for 1 to 4 wk; hyperthermia (37.8 to 39.5°C); vomiting (three infants); frank or occult blood in stools (three infants)

brain heart infusion broth for 48 h. Formalin was added (0.3%), and the cell suspensions were centrifuged at 12,000 × *g* for 20 min. The supernatant fluids were passaged at 4°C through a filter (molecular weight cutoff, 10,000; PM-10; Amicon Corp., Danvers, Mass.) to a retentate volume of 30 to 40 ml. The retentates were centrifuged (12,000 × *g* for 20 min), passed through a filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.), and plated onto TBA to ensure bacterial sterility. Portions of retentate from the two isolates of enterotoxigenic *B. fragilis* were heated (boiled for 30 min or heated at 70°C for 30 min), and all retentates were evaluated in the lamb LIL test. Unheated retentate (5 ml) from isolate 2-078382-3 was chromatographed on a column (74 by 2.5 cm) of Sephadex G-75 (void volume, 125 ml; Pharmacia Fine Chemicals, Piscataway, N.J.) at 4°C by ascending column chromatography. Fractions of 3 ml of eluant (0.05 M phosphate buffer; pH 7.7) were collected at a flow rate of 42 ml/h. Two column runs were made, and the fractions from the two runs were evaluated for enterotoxin activity in the lamb LIL test.

Rabbit studies. Brain heart infusion broth culture of *B. fragilis* (1 ml; 5×10^9 CFU) was injected directly into the ileum of a 1.8- to 2.2-kg New Zealand White rabbit in which the cecum had been ligated as described previously (11). A total of 15 rabbits were injected with one of two isolates of enterotoxigenic *B. fragilis*, and 8 rabbits were injected with one of two isolates of nonenterotoxigenic *B. fragilis* (see Table 2). A dose-response study was also done with 22 rabbits (see Table 3). In this study, 10-fold dilutions of a brain heart infusion broth culture of isolate 2-078382-3 were made. The number of CFUs was determined by plating appropriate dilutions of the challenge inoculum onto TBA plates. Rabbits were observed for clinical signs of enteric disease for 6 days postchallenge (p.c.). Tissues for histopathological examination were collected from five additional rabbits with ligated ceca that had been injected with 5×10^9 CFU of enterotoxigenic *B. fragilis* (isolates 2-078382-3, 20793-3, 20656-2-1) or with nonenterotoxigenic *B. fragilis* (isolates 20662 or 20669). The rabbits were euthanized 2 to 3 days p.c., and tissues were taken from three areas of the small intestine (5 cm from the cranial end of the duodenum, mid-jejunum, and 5 cm proximal from the caudal end of the ileum) and two areas of the colon (10 cm distal from the ileocecal junction and the rectum). The tissues were fixed in 10% buffered Formalin solution prior to histological examination. Three other diarrheic rabbits injected with enterotoxigenic *B. fragilis* and one clinically normal rabbit injected with nonenterotoxigenic *B. fragilis* were euthanized 48 h p.c. and cultured for *B. fragilis* at four approximately evenly spaced sites in both the small and large intestines. Isolates of *B. fragilis* were evaluated for enterotoxin production in the lamb LIL test.

RESULTS

Isolation of enterotoxigenic *B. fragilis*. *B. fragilis* was isolated from the feces of 6 of 10 diarrheic individuals in Montana and from 16 of 34 infants in Arizona. *B. fragilis* isolates from four individuals in Montana (first four cases listed in Table 1) and from four infants in Arizona were enterotoxigenic based on accumulation of fluid in the lamb LIL test (Fig. 1). Of the 32 pure cultures of *B. fragilis* from the 8 individuals (4 cultures from each individual) with enterotoxigenic *B. fragilis*, 28 cultures were enterotoxigenic. Individuals with enterotoxigenic *B. fragilis* had watery diarrhea, usually of 1 to 4 weeks in duration and often with mild to moderate intestinal cramping. Diarrheic infants with enterotoxigenic *B. fragilis* often had hyperthermia, vomiting, and frank or occult blood in the stools. The clinical disease associated with enterotoxigenic *B. fragilis* appeared to be self-limiting. No recognized enteric pathogens were detected in seven of the eight individuals with enterotoxigenic *B. fragilis*. One infant from Arizona was positive for enterotoxigenic *E. coli* that produced heat-stable enterotoxin only.

Enterotoxin studies. Filter (PM-10) retentates from two isolates of enterotoxigenic *B. fragilis* were positive in the lamb LIL test, whereas retentates from two isolates of nonenterotoxigenic *B. fragilis* were negative in the lamb LIL test. Enterotoxin activity was removed by heating the preparations at 70°C for 30 min. Following Sephadex G-75 column chromatography, enterotoxin activity was detected in column fractions 62 through 88, with the peak of activity

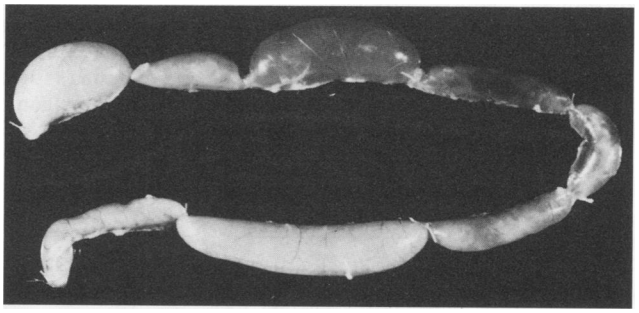

FIG. 1. A section of intestine from a lamb LIL test. Loops are numbered 1 through 8 starting at the upper left. Loops 1 and 7 were inoculated with viable cells of enterotoxigenic *B. fragilis* 2-078382-3 and 20656-2-1, respectively. Loops 2, 6, and 8 were inoculated with viable cells from different isolates of nonenterotoxigenic *B. fragilis*. Loops 3, 4, and 5 were inoculated with concentrated broth retentate, retentate boiled for 30 min, and retentate heated at 70°C for 30 min, respectively (from enterotoxigenic *B. fragilis* 20793-3). The volumes of fluid (in milliliters per centimeter of intestine) for loops 1, 3, and 7 were 0.6, 1.0, and 0.6, respectively. No measurable fluid was present in loops 2, 4, 5, 6, or 8.

(1 to 1.5 ml of fluid per cm of intestine) being at approximately fraction 79 (elution volume, 237 ml). By using either broth retentates or viable cells in the lamb LIL test, the accumulated fluid was cloudy, and a pseudomembrane formed which was composed primarily of fibrin and leukocytes.

Rabbit studies. The 15 rabbits injected into the ileum with enterotoxigenic *B. fragilis* developed enteric disease and died by 5 days p.c. (7 rabbits died 2 to 3 days p.c.), whereas the 8 rabbits given nonenterotoxigenic *B. fragilis* remained clinically normal (Table 2). Diarrhea (feces not pelleted) commonly occurred by 24 h p.c. in the rabbits. As the disease progressed, the feces became more mucoid and watery and often contained frank blood. Enterotoxigenic *B. fragilis* was isolated from the distal 30 cm (but not from the proximal 30 cm) of the small intestine and from the entire length of the colon in diarrheic rabbits. The duodenum and jejunum were normal histologically, whereas mild enteritis was observed in the ileum of infected rabbits. Moderate to severe necrotizing colitis was characteristic of diarrheic rabbits (Fig. 2). Nonenterotoxigenic *B. fragilis* was isolated from the ileum and colon of a rabbit inoculated with nonenterotoxigenic *B. fragilis*, and no intestinal lesions were detected. In the dose-response study (Table 3), as few as 5×10^3 CFU of enterotoxigenic *B. fragilis* caused fatal enteric disease. As the challenge dose was increased, the incidence of enteric disease increased. Nonfatal enteric disease was not observed.

DISCUSSION

Although enterotoxigenic *B. fragilis* has been isolated from the feces of several species of young, diarrheic livestock, this is the initial report of its isolation from diarrheic humans and of data indicating that human isolates of enterotoxigenic *B. fragilis* are enteropathogenic in an animal model. The bacterium was isolated from eight diarrheic individuals of widely different ages, and in seven of the eight individuals, recognized enteric pathogens were not detected. Diarrhea associated with enterotoxigenic *B. fragilis* was watery and usually of 1 to 4 weeks in duration.

Enterotoxigenic isolates of *B. fragilis* were highly virulent in the adult rabbit with ligated cecum, whereas isolates of nonenterotoxigenic *B. fragilis* were avirulent. In addition to the human isolates studied, we also evaluated 20 animal isolates of enterotoxigenic *B. fragilis* and 10 animal isolates of nonenterotoxigenic *B. fragilis* in rabbits. In all cases, the isolates of enterotoxigenic *B. fragilis* caused diarrhea (some apparently more so than others), and the nonenterotoxigenic *B. fragilis* isolates were avirulent (7; Myers, et al., in press; unpublished data). The virulence of enterotoxigenic *B. fragilis* in the rabbit with a ligated cecum (Table 3) appeared to be comparable with the virulence of *Vibrio cholerae* in rabbits in the RITARD test (rabbits with ligated ceca and with a reversible tie around the ileum) (10). At a dose of at least 10^6 CFU, *V. cholerae* caused fatal diarrhea in over 90% of rabbits in the RITARD test; the death rate was approximately 25% when 10^3 CFU was given. In earlier studies (unpublished data), we evaluated the virulence of enterotoxi-

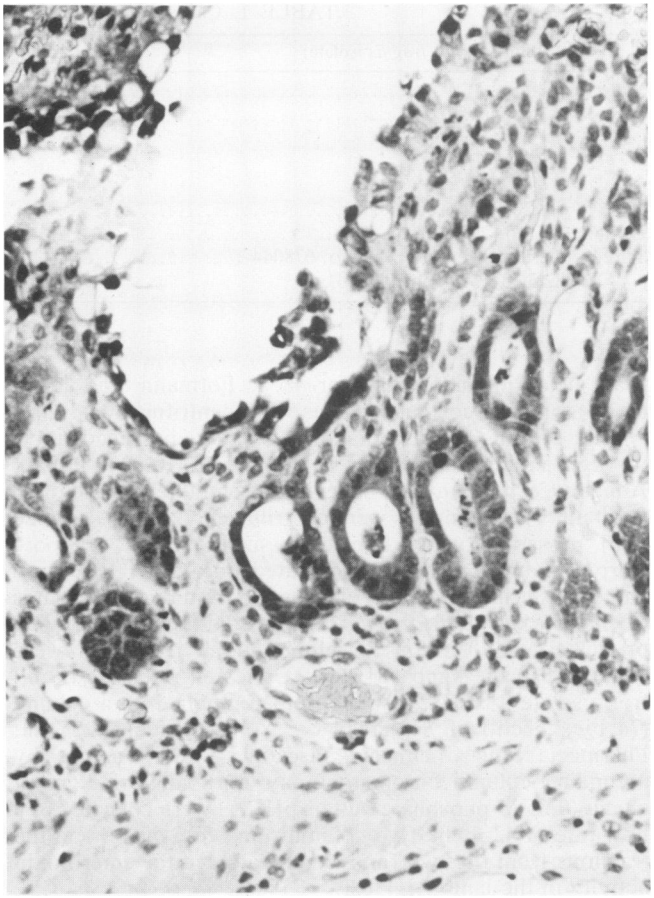

FIG. 2. Section of colon from a diarrheic, adult rabbit with ligated ceca previously inoculated with enterotoxigenic *B. fragilis* 2-078382-3. There is mucosal necrosis and crypt ectasia. Magnification, ×100.

TABLE 2. Response of rabbits with ligated ceca following intraileal inoculation with 5×10^9 CFU of *B. fragilis*

Challenge isolate	No. of rabbits	No. with diarrhea	Avg onset of diarrhea (h p.c.)	Avg duration (h) of diarrhea	No. that died
Enterotoxigenic *B. fragilis*					
20793-3	5	5	24	55	5
2-078382-3	10	10	27	65	10
Nonenterotoxigenic *B. fragilis*					
077351-3	4	0	0	0	0
283-2-1	4	0	0	0	0

TABLE 3. Response of rabbits with ligated ceca following intraileal injection of various doses of enterotoxigenic *B. fragilis* 2-078382-3

Challenge dose (CFU, 10^9)[a]	No. responding with fatal diarrhea[b]	Mean time (days) from challenge inoculation to death
0.5	3	2.2
0.05	3	4.0
0.005	2	4.5
0.0005	2	4.0
0.00005	1	4.0
0.000005	1	6.0
None	0	0

[a] Each challenge dose was administered to three rabbits, and four rabbits received no challenge dose.

[b] None of the rabbits had nonfatal diarrhea.

genic *B. fragilis* (5×10^9 CFU of isolate 2-078382-3) in nine rabbits using the RITARD test and in five rabbits without cecal or ileal ligation. The bacterium was avirulent in the five rabbits without ligation. Bacterial virulence was similar in rabbits with only cecal ligation as compared with rabbits in the RITARD test. In retrospect, since enterotoxigenic *B. fragilis* has a predilection for the colon, reversible ligation of the ileum would not appear to be indicated.

Enterotoxigenic *B. fragilis* elaborated a heat-labile enterotoxin into the broth during growth. Enterotoxin activity was also detected in the eluant buffer following gel filtration of broth retentates on Sephadex G-75. While the molecular weight of the enterotoxin was not determined in this study, in an earlier report (8) it was indicated that enterotoxin from a bovine isolate of enterotoxigenic *B. fragilis* had an apparent molecular weight of 19,500, as determined by Sephadex G-100 chromatography. Results of preliminary studies (5) have indicated that enterotoxin activity of bovine isolates of enterotoxigenic *B. fragilis* was not detectable in the infant mouse gastric test, the Y-1 mouse adrenal tumor cell assay, or in a gene probe assay for *E. coli* and *V. cholerae* enterotoxin. These negative results were obtained with viable cells of enterotoxigenic *B. fragilis* and with unconcentrated broth filtrates. In a recent study (unpublished data), we were also unable to satisfactorily detect the enterotoxin using broth retentates and viable cells in the rabbit ileal loop test. The enterotoxin appeared to cause fluid accumulation in some rabbits but not in others.

As is true of the established enteropathogens, we recently found that enterotoxigenic *B. fragilis* could be isolated from clinically normal individuals. In an ongoing study, enterotoxigenic *B. fragilis* thus far has been isolated from the feces of 19 of 123 (15.4%) diarrheic individuals and from 6 of 83 (7.2%) nondiarrheic, matched controls (unpublished data).

It was reported recently that anorexia and profuse, watery diarrhea occurred in four 1-day-old gnotobiotic pigs within 48 h after inoculation with 2×10^9 CFU of a porcine isolate of enterotoxigenic *B. fragilis* (J. R. Duimstra, J. E. Collins, L. L. Myers, and D. A. Benfield, Proceedings of the Conference of Research Workers in Animal Disease **67**:50, 1986 [abstr. 285]). Two gnotobiotic pigs inoculated with an isolate of nonenterotoxigenic *B. fragilis* from a pig with diarrhea remained clinically normal. As in the rabbit model, cellular changes included rounded up and detached enterocytes and occurred primarily in the large intestine of the gnotobiotic pig.

Enterotoxigenic and nonenterotoxigenic isolates of *B. fragilis* can be cultured from the feces of diarrheic and healthy individuals. These two groups of *B. fragilis* are presently indistinguishable from each other by standard in vitro bacteriological procedures and by serological analysis of heat-stable surface antigens (6). While isolates of nonenterotoxigenic *B. fragilis* appear to be avirulent for the intestinal tract, isolates of enterotoxigenic *B. fragilis* may, under the proper conditions, be enteropathogenic in animals and humans. Evidence suggestive of a significant causative role for enterotoxigenic *B. fragilis* in enteric disease include the common occurrence of enterotoxigenic *B. fragilis* in diarrheic individuals, the ability of the bacterium to produce an enterotoxin, and the marked enteropathogenicity of the bacterium in rabbit and pig models. We are presently conducting further studies regarding the possible role of enterotoxigenic *B. fragilis* in the enteric disease complex.

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