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# Association of Enterotoxigenic *Bacteroides fragilis* with Diarrheal Disease in Calves

*Bacteroides fragilis*, an obligate anaerobic bacterium found in high numbers in the intestinal tract of animals and humans, was recently associated with acute diarrheal disease in neonatal lambs [1]. Some ovine isolates of the bacterium had enterotoxin activity in the lamb and calf ligated intestinal loop (LIL) tests and caused diarrhea when given orally to newborn lambs. A selective medium (PINN medium) was developed for isolation of *B. fragilis* directly from feces [2].

Acute diarrheal disease in beef calves one to three weeks of age is a serious disease problem without effective preventive means. The condition is poorly understood etiologically and is associated with several potential etiologic agents, including rotavirus, coronavirus, and *Cryptosporidium* [3, 4]. Because the most prominent clinical sign often associated with the condition is profuse, watery diarrhea indicative of an enterotoxin (secretory) type of mediation, we conducted a study to evaluate the possible association of enterotoxigenic *B. fragilis* (ETBF) with the condition. If the organism produced a classical enterotoxin (a substance elaborated into the medium during bacterial growth that causes secretion of fluid into the intestinal tract) [5], such production could be assayed in the LIL test.

## Materials and Methods

*Collection of fecal samples.* A bulk fecal sample (1–10 g) was collected aerobically from each of 34 diarrheic beef calves on 10 ranches in Idaho and three ranches in Montana during the spring of 1984. Most of the calves were one to three weeks of age and had not been treated with antibacterial agents at the time of sample collection. Fecal samples were kept cool (at 4 C) until laboratory examination, usually one to four days after sample collection.

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*Laboratory examination of fecal samples.* Fecal specimens were evaluated for the presence of *Bacteroides* species and other potential enteric pathogens. *Bacteroides* species (primarily *B. fragilis*) were isolated as described previously [2]. In brief, fecal samples were streaked directly on PINN medium, which allowed growth of several species of *Bacteroides* but inhibited growth of most other enteric bacteria. After anaerobic growth (GasPak® anaerobe system; BBL Microbiology Systems, Cockeysville, Md) for 48 hr, two to four colonies with the characteristic colonial appearance of *B. fragilis* were subcultured to tryptose blood agar (Difco Laboratories, Detroit) to ensure purity and were presumptively speciated with use of catalase and indole tests and rhamnose, trehalose, and mannitol fermentation [6, 7]. Confirmatory testing of 29 isolates of *Bacteroides* species was done at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (Blacksburg).

Feces were evaluated for *Salmonella* species with use of tetrathionate broth (Difco) as an enrichment medium followed by plating of the broth on salmonella-shigella agar (Difco). Suspect colonies of *Salmonella* were identified by standard methods [8]. Fecal specimens were evaluated for rotavirus and coronavirus by direct electron microscopy [9]. Fecal smears stained with May-Grunwald-Giemsa stain were examined for *Cryptosporidium* [10]. In most cases fecal specimens were not evaluated for enterotoxigenic *Escherichia coli* because of the low frequency of occurrence and low virulence of the bacterium in calves over one week of age [3, 11].

*Enterotoxin studies.* *Bacteroides* species were grown anaerobically for 48 hr in tryptic soy broth (Difco), in brain-heart infusion broth (Difco) without or with 0.5% yeast extract (Difco), or as confluent growth on tryptose blood agar plates (15 × 100 mm). The enterotoxin activity of all *Bacteroides* isolates was assessed with use of viable whole cells in the lamb or calf LIL test [1, 12, 13]. Broth cultures, ½ and 2 ml, were used in the lamb and calf tests, respectively. About one-half of the confluent growth washed off a tryptose blood agar plate was used in the lamb test, and all the growth was used in the calf test. Intestinal loop scores of 4, 2, and 0 indicated loops tightly distended with fluid, about one-half filled, or essentially devoid of fluid, respectively. Enterotoxin activity was considered to be present in any loop with a score at least two numbers greater than any adjacent loop.

To determine whether *B. fragilis* produced a classical enterotoxin, we cultured 10 isolates of ETBF and three isolates without enterotoxin activity in 800 ml of brain-heart infusion broth. After anaerobic growth for 48 hr, the cells were centrifuged at 12,000 *g* for 20 min, and the supernatant was passaged at 4 or 22 C through a stirred ultrafiltration unit (model 2000; Amicon Corp., Danvers,

Mass) to a retentate volume of 35–40 ml. The filters used had molecular weight cut-offs of $3 \times 10^4$ (PM-30; Amicon), $1 \times 10^4$ (type C, 10K ultrafiltration membrane; Ultra/Por, Los Angeles), or $1 \times 10^3$ (UM-2; Amicon). After ultrafiltration the retentate was centrifuged at 12,000 g for 20 min, passaged through a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass), and plated on tryptose blood agar to ensure bacterial sterility. The retentate was then evaluated in the calf or lamb LIL test. Heat stability of the enterotoxin activity in selected retentate was assessed by heating a portion of the retentate in a water bath at 60, 70, or 80 C for 30 min. Aliquots of 2 and 4 ml of retentate were evaluated for enterotoxin activity in the lamb or calf LIL tests, respectively.

The enterotoxin was chromatographed on a column of Sephadex® G-100 (Pharmacia Fine Chemicals, Piscataway, NJ), and its elution volume was compared with that of four proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, and bovine serum albumin) of known molecular weight. Descending column chromatography was conducted at 4 C with use of a 2.5 × 75-cm column and a flow rate of 24 ml/hr. Two-milliliter fractions of eluant (0.05 *M* phosphate buffer, pH 7.7) were collected. The enterotoxin solution (4 ml) placed on the column was the same as the retentate (described above) used in the LIL test with the exception that the brain-heart infusion broth was prefiltered (type C, 10K ultrafiltration membrane) before growth of ETBF and the supernatant was concentrated 35-fold with the same filter. The elution volume of the enterotoxin was determined by analysis of 0.5 ml of each column fraction in the lamb LIL test. The elution volume of the four standard proteins was determined based on $A_{280}$. A standard curve was plotted of the log of the molecular weights vs. the elution volume. The molecular weight of the enterotoxin was estimated from this standard curve.

*Infant mouse gastric test.* The infant mouse gastric test [14] for enterotoxin activity was used to evaluate two viable cultures of ETBF and two concentrated broth retentates, all of which were previously shown by the lamb LIL test to be enterotoxigenic. A volume of 0.15 ml of each sample was inoculated orally into four three-day-old mice. The mice were killed after 3.5 hr, and the amount of fluid accumulation was estimated from the ratio of the weight of the intestinal tract to the weight of the remainder of the mouse. Viable cells of enterotoxigenic *E. coli* strain B41 were used as a positive control.

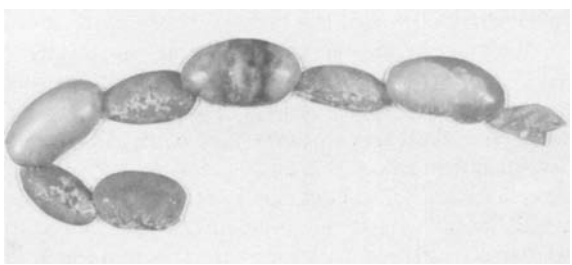

**Figure 1.** A section of ligated intestine from a calf LIL test. Loops 1, 3, 5, and 7 (starting at top right) were each inoculated with broth from a different isolate of ETBF concentrated with a 10K filter. Loops 2, 4, and 6 were, in each case, inoculated with broth from the preceding loop heated at 70 C for 30 min. The amount of fluid, in ml/cm of intestine, for loops 1–7 was 3.2, 0.7, 3.6, 1.2, 3.2, 0.7, and 2.1, respectively.

## Results

*Presence of ETBF in diarrheic calves.* ETBF was isolated from the feces of 15 of 34 diarrheic beef calves, representing 10 of the 13 herds studied. Of the 120 isolates of *B. fragilis* obtained, 38 had enterotoxin activity in the calf or lamb LIL test (figures 1 and 2). The medium used (tryptic soy broth, brain-heart infusion broth alone or with yeast, or tryptose blood agar) for growth of *B. fragilis* did not affect results in the test. The calf and lamb tests gave comparable results. The test score (range) of fluid accumulation in ml/cm of intestine for the lamb LIL test was 4 (1.2–2.0), 3 (0.7–1.2), 2 (0.3–0.7), and 1 or 0 (<0.3). Comparable values for the calf test were 4 (2.5–3.6), 3 (1.5–2.5), 2 (1.0–1.5), and 1 or 0 (<1). In eight of the 15 calves, ETBF was the only potential enteric pathogen detected. There were dual infections with ETBF plus rotavi-

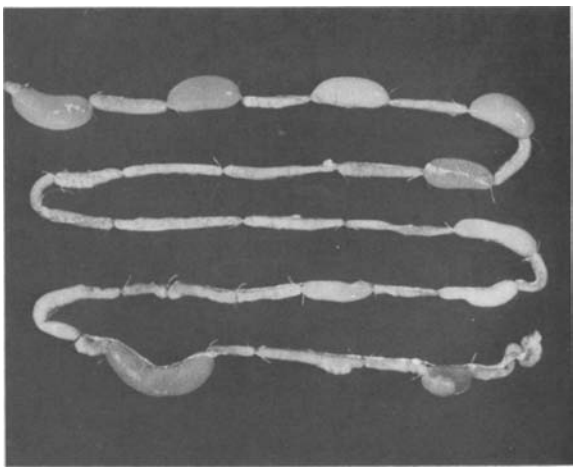

**Figure 2.** Results of a lamb LIL test. Loop 1 (top left) and loop 33 (lower right) were inoculated with enterotoxigenic *E. coli* strain B41. Even-numbered loops were not inoculated. Loop 29 (positive loop, lower left) was inoculated with enterotoxin from ETBF isolate 20604 concentrated with a 10K filter. The other loops were inoculated with viable cultures of *B. fragilis*. The amount of fluid, in ml/cm of intestine, for loops 1, 3, 5, 7, 9, 19, 21, 23, 27, 29, and 33 was 1.9, 1.8, 1.2, 1.7, 1.5, 0.7, 0.3, 0.7, 0.3, 1.5, and 1.0, respectively.

rus in two calves, ETBF plus coronavirus in two calves, ETBF plus *Cryptosporidium* in two calves, and ETBF plus *Salmonella* species in one calf. All nine isolates of *Bacteroides thetaiotaomicron* and all three isolates of the *Bacteroides* 3452A group [15] were nonenterotoxigenic.

*Enterotoxin studies.* Enterotoxin activity was detected in broth retentates after ultrafiltration from all 10 isolates of ETBF studied in the calf and lamb LIL tests, whereas no activity was detected in broth retentates from three isolates of *B. fragilis* without enterotoxin activity. Activity was retained after ultrafiltration with the UM-2 or the 10K filters but not with the PM-30 filter. The temperature (4 or 22 C) of filtration had no detectable effect on enterotoxin activity. Enterotoxin activity was destroyed by heating broth retentates at 70 or 80 C for 30 min but not at 60 C for 30 min. Activity was retained after aerobic storage of broth retentates at 4 or −5 C for three months, the maximum length of storage tested. Enterotoxin activity was not detected in unconcentrated nutrient broth after growth of ETBF.

Enterotoxin activity was detected with the LIL test in about six fractions from the Sephadex G-100 column. The elution volume for the enterotoxin was estimated as the volume of eluant in the center of the positive fractions. With use of the standard curve established with the four proteins of known molecular weight, the molecular weight of the enterotoxin was ~19,500.

The infant mouse gastric test gave negative results for enterotoxin activity with viable cells of ETBF and broth retentates. Intestine/body weight ratios were 0.055–0.065 for ETBF and the retentates and ~0.13 for enterotoxigenic *E. coli* strain B41.

## Discussion

This study established that some calf isolates of *B. fragilis* produced a heat-labile, classical enterotoxin and that ETBF was prevalent in the feces of young calves with diarrhea. The bacterium was often found alone but was also found randomly in dual infections with other potential enteric pathogens. Enterotoxin activity was demonstrated with use of the calf and lamb LIL tests but not infant mouse gastric test. Five bovine and two ovine isolates of ETBF were inactive in the porcine LIL test (E. M. Kohler, personal communication), and both retentates from two bovine isolates of ETBF gave negative results in the Y-1 mouse adrenal tumor cell assay (Ginger Scheuerman, personal communication). The use of PINN medium markedly facilitated the isolation of *B. fragilis* from feces. The medium inhibited the growth and spread of *Proteus* species on tryptose blood agar plates and prevented growth of a number of other intestinal bacteria. The characteristic mottled or swirled internal appearance of colonies of *B. fragilis* facilitated their selection from the medium. Isolates of ETBF and non-ETBF were identical in appearance on PINN medium. The two types of *B. fragilis* often occurred together, and it is likely that ETBF was missed in some calves because of failure to select an enterotoxigenic colony. It is also possible that some strains of ETBF did not grow on PINN medium because of the presence of antibacterial agents in the medium.

An attempt was made (authors' unpublished observation) to establish the virulence of ETBF. About $10^{12}$ viable cells of ETBF were administered orally to nine conventional dairy calves. Six of the nine calves developed diarrhea, usually within 24–48 hr after challenge. In most cases the diarrhea lasted ~48 hr and ceased spontaneously. However, most of the diarrheic calves were also infected with *Cryptosporidium* or rotavirus, thus precluding determination of the virulence of ETBF. Uninoculated control calves were not included in the study.

The enterotoxin molecule from ETBF is relatively small compared with other heat-labile bacterial enterotoxins. Its activity is not detected with use of Y-1 adrenal cells, the infant mouse gastric test, the pig LIL test, or a gene probe assay [1] for detection of *E. coli/Vibrio cholerae*-like enterotoxin. The molecular weight determined for *B. fragilis* enterotoxin (reported herein) may be somewhat inaccurate because (*1*) it was determined by comparison with the behavior of globular proteins on Sephadex, whereas the enterotoxin may not be a globular protein, and (*2*) the LIL test was used for determination of the elution volume of the enterotoxin. A more precise determination of molecular weight awaits a better understanding of the enterotoxin's molecular nature and a quantitative test for its detection.

*Bacteroides fragilis* is present in relatively high numbers in the intestinal tract of normal animals and humans. Some isolates produce a classical enterotoxin, whereas others do not. The bacterium possesses those attributes (aerotolerance, good intestinal colonizing ability, and enterotoxigenicity) consistent with the hypothesis that it is an enteric pathogen. Additional studies are needed to explore further the possible role of ETBF in enteric disease of humans and animals.

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