**Detection of Bacteroides fragilis Enterotoxin Gene by PCR**

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*Bacteroides fragilis* constitutes about 1% of the bacterial flora in intestines of normal humans. Enterotoxigenic strains of *B. fragilis* have been associated with diarrheal diseases in humans and animals. The enterotoxin produced by these isolates induces fluid changes in ligated intestinal loops and an in vitro cytotoxic response in HT-29 cells. We developed a nested PCR to detect the enterotoxin gene of *B. fragilis* in stool specimens. After DNA extraction, a 367-bp fragment was amplified with two outer primers. The amplicon from this reaction was subjected to a second round of amplification with a set of internal primers. With these inner primers, a 290-bp DNA fragment was obtained which was confirmed as part of the *B. fragilis* enterotoxin gene by Southern blotting with a nonradioactive internal probe and a chemiluminescence system. By this approach, *B. fragilis* enterotoxin gene sequences were detected in eight known enterotoxigenic human isolates and nine enterotoxigenic horse isolates. No amplification products were obtained from DNA extracted from 28 nonenterotoxigenic *B. fragilis* isolates or *B. distasonis* or *B. thetaiotaomicron*. The sensitivity of this assay allowed us to detect as little as 1 pg of enterotoxin DNA sequences or 100 to 1,000 cells of enterotoxigenic *B. fragilis*/g of stool. Enterotoxin production of all isolates was confirmed in vitro in HT-29 cells. A 100% correlation was obtained between enterotoxin detection by cytotoxin assay and the nested PCR assay. This rapid and sensitive assay can be used to identify enterotoxigenic *B. fragilis* and may be used clinically to determine the role of *B. fragilis* in diarrheal diseases.

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**MATERIALS AND METHODS**

**Bacterial strains and culture media.** *B. fragilis* ATCC 43858, 43859, and 43860 were obtained from the American Type Culture Collection. *B. fragilis* W1 and W2 were kindly provided by F. Meisel-Mikolajczyk from the Medical Academy of Warsaw, Poland. These five isolates are enterotoxigenic in humans. Fifteen other isolates obtained from patients with diarrhea were also analyzed in this study. Twenty-five *B. fragilis* isolates were obtained from the University of California, Davis, School of Veterinary Medicine, and were recovered from horses with diarrhea. *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *E. coli*, and *C. difficile* were obtained from the Clinical Laboratories at the University of California, Davis Medical Center, Sacramento. *Clostridium difficile* P-224 is a toxigenic isolate from our culture collection. All *B. fragilis* group isolates were grown on the selective medium *Bacteroides* bile esculin agar (Anaerobe Systems, San Jose, Calif.) for 36 to 48 h at 37°C under anaerobic conditions. Isolates were confirmed to be *B. fragilis* if they were catalase positive and indole negative. *E. coli* was grown on *Brucella* anaerobic plates. *C. difficile* was grown in the selective medium cycloserine-cefoxitin-fructose agar as described elsewhere (15).

**DNA extraction from pure cultures and stool specimens.** DNA was extracted from colony cultures of *B. fragilis* as described previously (15). DNA was extracted from spiked stools by a modification of the methods of Balabat et al. and Kato et al. (1, 3). Briefly, 1 g of stool which tested negative for *B. fragilis* was spiked with ETBF at concentrations ranging from 10 to 10⁶ cells/g of stool. For DNA extraction, 100 mg of stool was suspended in 400 μl of TES buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl) and centrifuged at 2,000 × g for 5 min to remove large particles. The supernatant was then centrifuged at 7,000 × g for 5 min to pellet bacterial cells. The pellet was resuspended in TES plus 25% sucrose and incubated with 5 μl of lysozyme (100 mg/ml) for 1 h at 37°C. The suspension was centrifuged at 7,000 × g, and the pellet was resuspended in TES containing 0.8% Sarkosyl and 100 μg of protease K/ml and incubated at 60°C for 2 h. After digestion with protease K, the supernatant was extracted twice with phenol-chloroform-isooamyl alcohol (24:24:1), and the DNA was precipitated with 2 volumes of absolute ethanol.

**DNA amplification by PCR.** The sequences of the oligonucleotide primers and probe used in this study are listed in Table 1. These oligonucleotides were designed according to the *B. fragilis* enterotoxin published sequence (4). A nested PCR approach was used to amplify a 290-bp fragment of the ETBF gene in stool specimens. The PCR method of Mullis and Faloona was used for amplification with the thermostable DNA polymerase (Taq polymerase). The reaction mixtures were prepared in 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM MgCl₂, 100 μg of bovine serum albumin per ml [pH 8.4]) and contained per reaction 20 pmol of the respective primers, 0.1 mM concentrations of each of the dNTPs, and 1 U of recombinant DNA polymerase (Taq polymerase), and 10 μl of purified DNA from stools. For the second round of
from horses with diarrhea. Amplification with the inner primers (RS-1 and RS-2), we amplified the expected size of 367-bp DNA fragment in eight known enterotoxigenic strains of outer primers (RS-3 and RS-4), we amplified the expected size of 367-bp DNA fragment in eight known enterotoxigenic strains of enterotoxigenic B. fragilis. Amplification of enterotoxigenic strains of B. fragilis for amplification of the enterotoxin gene of B. fragilis. Amplification of enterotoxigenic strains of B. fragilis.

**Detection of amplified products.** Amplification products were visualized by running 9 μl of the reaction mixture in 2% agarose gels in Tris-borate–EDTA buffer. Size markers in all gels were from the 123-bp DNA ladder (Bethesda Research Laboratories, Grand Island, N.Y.). Gels were run at a constant 110 V for 90 min, stained in an ethidium bromide solution (0.5 μg/ml) for 30 min, destained for 30 min, and photographed under UV light with a Land camera (Polaroid, Cambridge, Mass.).

**Southern blot analysis.** PCR products were confirmed as B. fragilis enterotoxin gene sequences by internal probe hybridization. A nonradioactive method with horseradish peroxidase, a biotin-labeled oligonucleotide (RS-7), and an enzyme chemiluminescence detection system (Amersham, Arlington Heights, Ill.) were used as previously described (2).

**Sensitivity of PCR in detecting ETBF.** For the sensitivity experiment in stool specimens, a nested PCR approach was used as described in Materials and Methods. The sensitivity of our assay in detecting ETBF was determined in two sets of experiments. In the first experiment, ETBF at concentrations ranging from 10 to 10⁶ cells was inoculated into 1 g of stool from a healthy person. Using a nested PCR approach, we detected 100 to 1,000 cells of ETBF per g of stool (Table 2 and Fig. 2). No amplification product was detected in DNA extracted from the unspiked stool by the nested PCR method for B. fragilis. The 16S rRNA gene of enteric bacteria was amplifiable in this specimen, indicating the lack of inhibitors of Taq polymerase in the sample.

**Sensitivity of the nested PCR was determined with ETBF DNA ranging in concentrations from 1 pg to 100 ng. In this**

<table>
<thead>
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<tr>
<td><strong>Outer primers</strong></td>
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<tr>
<td>RS-3</td>
<td>TGA AGT TAG TGC CCA GAT GCA GG</td>
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<td>RS-4</td>
<td>GCT CAG CGC CCA GTA TAT GAC C</td>
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<td>RS-1</td>
<td>TGC GGC GAA CTC GGT TAA TGC</td>
<td>290</td>
</tr>
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<td>RS-2</td>
<td>AGC TGG GTT GTA GAC GAC ACG C</td>
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<tr>
<td><strong>Probe RS-7</strong></td>
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<td></td>
<td>AGC CCA AAG AGG GAG TGG AAG G</td>
<td>290</td>
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**RESULTS**

**Amplification of enterotoxigenic strains of B. fragilis.** A PCR assay was developed to detect ETBF. We designed and tested two sets of primers for later use in a nested assay. With a pair of outer primers (RS-3 and RS-4), we amplified the expected 367-bp DNA fragment in eight known enterotoxigenic strains of B. fragilis isolated from humans and nine strains isolated from horses with diarrhea. Amplification with the inner primers (RS-1 and RS-2) yielded the expected 290-bp DNA fragment (Fig. 1A). Nonspecific amplification products were observed with DNA from 28 nonenterotoxigenic isolates (these isolates did not show any cytotoxic activity in HT-29 cells). Confirmation of specificity of the product was by Southern blot analysis with a biotin-labeled internal probe (RS-7). As seen in Fig. 1B, only DNA amplification products from toxigenic strains hybridized with the probe. None of the amplicons observed with the nonenterotoxigenic isolates probe positive.

**FIG. 1. Ethidium-bromide-stained 2% agarose gel (A) and Southern blot (B)**

- **A:** Amplification products were scored as visible bands ranging from 10 to 10⁶ cells in B. fragilis. The sensitivity of the nested PCR in detecting ETBF was determined with ETBF DNA ranging in concentrations from 1 pg to 100 ng. This

**amplifications, the reaction mixture was prepared as described above, except that the inner primers RS-1 and RS-2 were used and 5 μl of the amplified product from the first PCR was used as the source of DNA. For amplification of DNA from pure cultures, 5 μl of the DNA preparation was amplified with the external (RS-3 and RS-4) or internal (RS-1 and RS-2) primers as described above. The reaction mixtures were covered with 150 μl of mineral oil to prevent evaporation. The PCR profile included a denaturing step at 95°C for 30 s, followed by annealing of the primers at 60°C for 30 s, with extension at 72°C for 30 s. For the outer PCR, amplification was done for 35 cycles in a thermal cycler (MJ Research). Amplification with the inner primers was done for 30 cycles. Negative controls consisted of a blank containing all PCR reagents but no DNA. As control for amplifiable DNA in the stool specimen, primers targeting the 16S rRNA of enteric bacteria were used as described elsewhere (3).

- **B:** Amplification products were scored as visible bands ranging from 10 to 10⁶ cells in B. fragilis. The sensitivity of the nested PCR in detecting ETBF was determined with ETBF DNA ranging in concentrations from 1 pg to 100 ng. This

**TABLE 1. Sequences of oligonucleotide primers and probe used for amplification of the enterotoxin gene of B. fragilis**

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experiment, as little as 1 pg of enterotoxigenic DNA was detected by nested PCR (Table 2).

**Specificity of the PCR.** To determine the specificity of the PCR, amplification reactions were carried out with DNA from other species in the *B. fragilis* group, *E. coli*, and toxigenic *C. difficile*. As shown in Fig. 3A, no amplification products of the expected size were obtained with DNA from *B. distasonis*, *B. ovatus*, *B. uniformis*, and *B. thetaiotaomicron*, *E. coli*, or toxigenic *C. difficile* after amplification with the primers RS-3 and RS-4. DNA extracted from all these species remained negative when probed with the internal probe (Fig. 3B).

**Correlation between PCR and enterotoxin assay.** We obtained a 100% correlation between PCR and the enterotoxin assay in HT-29 cells. All the isolates positive by PCR produced enterotoxin (data not shown).

**DISCUSSION**

*B. fragilis* is emerging as an etiologic agent of diarrhea in animals and in humans (7–9, 12, 14). Although *B. fragilis* constitutes about 1 to 2% of the normal intestinal flora, it has been associated with extraintestinal infections such as abscesses, soft-tissue infections, and bacteremias (5). In a recent communication, Pantosi et al. reported the isolation of ETBF from the stools of healthy children and adults as well as from those of children and adults with diarrhea (11). These investigators, however, were able to detect *B. fragilis* enterotoxin directly in only a portion of the stool specimens positive for ETBF by culture. Detection of *B. fragilis* enterotoxin in stools is dependent on amount of toxin produced, sensitivity of the assay, and
stability of the toxin, which is susceptible to degradation by proteases (18).

In this study, we described a rapid, sensitive, and specific method for detection of ETBF directly in stool specimens. Sensitivity of the nested PCR allowed us to detect as little as 1 pg of enterotoxigenic DNA or 100 to 1,000 cells of ETBF per g of stool. Currently, identification of ETBF isolates is done by culturing in selective medium (Bacteroides bile esculin) and testing the isolates for the presence of enterotoxin by either a cytotoxicity assay in HT-29 cells or the lamb ileal loop test (17). Culturing on selective medium requires the presence of 10⁶ CFU, although recovery of isolates from specimens is dependent on time of culturing from obtainment of the fecal sample (13). The cytotoxicity assay in HT-29 cells has been reported to have 89% sensitive compared to the lamb ileal loop test (19). The latter biological assay is obviously more expensive and labor-intensive. Specific DNA amplification of ETBF from stool specimens eliminates the need for culturing the organism and for further biochemical tests for identification. We obtained a 100% correlation between PCR and the enterotoxin assay in HT-29 cells. Thus, a single test can be used to detect and identify enterotoxigenic isolates of B. fragilis. Considering the time required to culture, purify the isolate, and test for enterotoxin production in vitro with a tissue culture assay (5 to 6 days), the PCR assay is a rapid test. The time required to carry out this assay, approximately 48 to 72 h, can be shortened as the assay is refined. The clinical significance of ETBF as a cause of diarrhea in humans is not clear. In two separate studies, ETBF has been significantly associated with diarrhea in children >1 year of age in Bangladesh and in an Apache population in the United States (12, 14). In a recent study, a high rate of carriage of ETBF was found in healthy subjects and in subjects with diarrhea in Italy, both children and adults (11). We believe our PCR assay is of clinical importance and significance, as it will detect ETBF in patient samples in which the organism may be undetected by the conventional tests. At present, we continue to optimize and refine this assay, including screening of new primers. This assay may contribute to the elucidation of the role and epidemiology of ETBF in diarrheal and intestinal infectious diseases in humans. Studies are in progress in our laboratory to determine the incidence rates of B. fragilis in several forms of diarrheal diseases.

ACKNOWLEDGMENTS

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REFERENCES