Detection of Enterotoxigenic Bacteroides fragilis by PCR

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Strains of enterotoxigenic Bacteroides fragilis (ETBF) are associated with diarrhea in young farm animals and, at least in particular settings, in children. Enterotoxin production by ETBF is currently detected by a tissue culture assay with HT-29 cells. We have developed a PCR assay based on the detection of the enterotoxin gene to identify ETBF in culture and in stool samples. Overall, 113 bacterial strains were examined, including 3 B. fragilis reference strains, 75 B. fragilis isolates (comprising 40 ETBF isolates), 20 Bacteroides spp. other than B. fragilis, and 15 strains belonging to other genera. Complete agreement was found between the results of the tissue culture assay and those of the PCR for our strains. PCR was also used to detect ETBF directly in fecal samples. Stools from two healthy volunteers were spiked with known numbers of ETBF and were processed by three different methods. A culture method, which required inoculation of the stools on selective plates and the collection of the whole bacterial growth (“sweeps”), was found to be the most sensitive. PCR performed with the plate sweeps yielded amplification products with a detection limit of 105 to 104 CFU/g of feces. By this method 18 samples of diarrheic stools (10 positive and 8 negative for ETBF) were examined. The results of the PCR were in accordance with the culture results in all cases. The proposed PCR assay represents a diagnostic tool for the rapid identification of ETBF in culture as well as in fecal samples.

In recent years, a number of studies have focused on the possible role of Bacteroides fragilis in intestinal diseases of animals and humans. More than a decade ago Myers and coworkers (11, 12) found that some strains of B. fragilis were associated with diarrheal diseases in young farm animals and that these strains were able to produce a factor with enterotoxigenic activity; thereafter, such strains were designated entero-otoxigenic B. fragilis (ETBF). Subsequent studies showed that in particular settings the isolation of ETBF strains from stools correlated significantly with diarrhea in children from 1 to 5 years of age (17–19). Recently, we found that in Italy the rate of ETBF carriage is high both in adults and in children, regardless of whether diarrhea is present (16). ETBF strains are not restricted to the gut, but they are found at a high rate among B. fragilis isolates isolated from extraintestinal infections (15). Some studies have suggested that the enterotoxin can function as an important virulence factor for B. fragilis, possibly both inside and outside the intestinal lumen. The enterotoxin, which has been identified as a zinc-dependent metalloprotease (9), has been shown to produce extensive tissue damage in the intestinal mucosa in vivo (14), to increase bacterial internalization by enterocytes (28), and to modify the actin cytoskeleton of sensitive cells (3, 7). Therefore, more studies to define the epidemiology and pathogenic role of ETBF are necessary.

The production of enterotoxin was originally demonstrated through the ability of supernatants of broth cultures of ETBF strains to elicit fluid accumulation in the ligated intestinal loops of large animals (lamb or calves) (11, 12). Subsequently, ETBF supernatants were demonstrated to be cytotoxic for a cultured cell line derived from a human colon carcinoma, HT-29 (25, 27); in this cell line, the enterotoxin causes revers-
Enterotoxin production. Enterotoxin production by the strains was assayed by a cytotoxicity assay with HT-29 cells as described previously (15). Bacteroides spp. and C. difficile strains were incubated in supplemented brain heart infusion broth and were incubated overnight in an anaerobic cabinet (Microflow Anaerobic System, MDH, Andover, United Kingdom). Aerobic and facultative strains were grown in the same medium and were incubated overnight in air at 37°C, with the only exception being L. pneumophila strains, which were grown in nutrient broth containing Legionella BCYE-a Growth Supplement (Oxoid SpA, Milan, Italy) in 0.25% CO2 for 48 h. Tenfold dilutions of the broth supernatants were prepared. Each sample was inoculated in duplicate wells of a microtiter plate containing semisolid HT-29 cells. Rounding of the cells after 4 h of incubation was considered positive for the presence of the enterotoxin (15).

Preparation of bacterial strains for PCR analysis. B. fragilis strains were grown on Columbia agar (Oxoid) plates supplemented with 5% sheep blood–coal yeast extract agar plates (Oxoid) for 24 h. Each sample was obtained from either of two healthy adult volunteers (donor 1 and donor 2). The presence of ETBF in stools was ruled out by previously described methods (16). A suspension of strain VPI 13784 was prepared as described above to a cell density corresponding to an optical density at 600 nm of 1 (approximately 2 × 109 CFU/ml). Serial 10-fold dilutions were prepared and added to preweighed samples from the two donors in order to attain concentrations ranging from 109 to 100 CFU of feces. A nonspiked sample from each donor was also included. For PCR analysis of feces, the spiked fecal samples were processed by three different methods. (i) The first was a rapid purification method described by Arzese and coworkers (1). Briefly, a diluted suspension of feces (50 mg in 1 ml of distilled water) was heated at 100°C for 10 min, digested with proteinase K and pronase (0.5 mg/ml of each enzyme; Sigma Chemical Co.) at 56°C for 90 min, heated again at 100°C for 5 min, and finally, centrifuged at 10,000 × g for 5 min. The supernatant was used as a template. (ii) The second method was extraction of DNA with a commercially available kit (QiAamp Tissue Kit; Qiagen GmbH, Hilden, Germany). This procedure is based on adsorption of DNA to a silicon membrane. The protocol used was essentially that recommended by the manufacturer for the processing of the tissue, with the difference being that 10 mg of stools instead of 25 mg of tissue was processed for extraction of DNA and that the final elution volume of DNA was 200 µl. (iii) Finally, a culture method was used. One loopful of each reconstructed fecal sample was plated onto two B. cereus (BBE) agar plates, a selective medium for Bacteroides (23), and the plates were incubated in an anaerobic cabinet at 37°C. One set of plates was processed after 24 h of incubation. One plate was then inoculated with 1 ml of a 10-fold dilution of each reconstructed fecal sample and was then incubated anaerobically at 37°C for 48 h. Whole bacterial growth was removed with a sterile spreader, transferred to a sterile microcentrifuge tube, and centrifuged at 13,000 × g for 5 min. The pellet was resuspended in 500 µl of ultrapure water. This preparation was processed in a manner identical to that used for the purification of the pure cultures. Swipes were kept frozen at −20°C until PCR was performed. The second set of plates was processed after 48 h of incubation.

Preparation of fecal samples from patients with diarrhea for PCR analysis. Fecal samples from patients with diarrhea were purified by the method described only. These samples were collected in previous (16) and ongoing studies of the prevalence of ETBF in Italy and comprised a total of 18 samples, 5 from children and 13 from adults. The samples had been examined for the presence of ETBF and other intestinal pathogens by standard methods (16) and were kept frozen at −80°C until they were processed for PCR. ETBF was detected in 10 samples by conventional techniques. The other diarrheal samples yielded C. difficile, two patients), Salmonella typhimurium (two patients), Campylobacter spp. (two patients), and no known pathogens (two patients). The fecal samples were thawed, seeded onto BBE agar plates, and incubated for 48 to 72 h until the bacterial growth was visible. The swipes were obtained from the plates and processed for PCR as mentioned above for the spiked samples.

PCR assay. The primers used were derived from the published sequence of the enterotoxin gene (9) with the aid of the computer program Oligo, version 4.0 (National Biosciences Inc., Plymouth, United Kingdom). Forward primer BFI (5′-dGACGGTGATGTGTTGTGTGCTGAGAGA-3′) and backward primer BFE (5′-dATCCCTGAATTTATTGTTATACCCAGTA-3′) were synthesized by Pharmacia Biotech (Uppsala, Sweden). The expected amplification product of 294 bp spans bases 9 to 303 of the published partial enterotoxin gene sequence, upstream of the zinc-binding site (9). The reaction mixture contained 2.5 µl of DNA template (100 µl of each primer, DynaZyme II DNA polymerase (1 U; Finzyme), and 10 µl of DNA template in a final volume of 100 µl of enzyme buffer containing 1.5 mM MgCl2 (Finzyme). Samples were subjected to 35 amplification cycles carried out in a DNA Thermal Cycler (Mastercycler 5330; Eppendorf, GmbH, Milan, Italy). Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, and 1 min of extension at 72°C. The last cycle was followed by 5 min of extension at 72°C. For fecal samples, the 35 cycles were preceded by 5 min of denaturation at 94°C. A negative control (containing water instead of template DNA) and a positive control (containing reference ETBF DNA template) were run in each PCR experiment. Amplified DNA (10 µl) was subjected to electrophoresis in a 1.6% agarose gel containing 0.5 µg of ethidium bromide per ml at 90 mV for 70 min. Molecular mass markers (Marker V; Boehringer Mannheim GmbH, Mannheim, Germany) were run concurrently. The DNA bands were visualized and photographed under UV light.

RESULTS

PCR amplification of DNA from bacterial strains. We obtained the anticipated 294-bp amplification product from reference ETBF strains VPI 13784 and ATCC 43858 as well as from the other 40 ETBF strains isolated both from feces and from clinical samples. The type strain NCTC 9343 was negative in the cell culture assay and negative in the PCR test (Fig. 1). We never obtained nonspecific products, that is, bands with molecular sizes different from those obtained with the control ETBF strains. None of the 35 nonenterotoxigenic B. fragilis isolates yielded any amplification product. The other strains tested, belonging to Bacteroides spp. other than B. fragilis and to other bacterial genera, were always negative in both the HT-29 assay and the PCR assay. Overall, we found complete agreement between the tissue culture assay and the PCR for all the strains examined (Table 1).

PCR amplification of DNA from spiked stools. Three different methods of treatment of stools, including a rapid method, a method with a commercially available kit, and a culture method, were compared. When DNA extracted by the rapid method was used for PCR, no amplification product was obtained from any of the samples tested. The DNA preparations contained a PCR inhibitor, as indicated by the lack of amplification when 1 µl of positive control DNA was added to the test samples (data not shown). Therefore, we tried diluting the template DNA 1:10 and 1:100 in distilled water prior to PCR, but these diluted samples also did not yield any amplification product. The assay was not improved when a first step of differential centrifugation was included, when Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) was added during boiling, or when the amount of thermostable DNA polymerase was increased from 1 to 2.5 U per reaction tube (data not shown). By the second method, with the commercially available kit, we did not obtain any amplification product from the samples when we added 10 µl of the preparation as a template to the reaction mixture. When we added only 1 µl as template (10-fold dilution of the template DNA), however, samples from both donors spiked with 109 to 108 CFU/g of feces yielded amplification products, although the intensities of the bands

FIG. 1. Agarose gel electrophoresis of PCR products from Bacteroides spp. for the detection of ETBF. Lanes: 1, B. fragilis VPI 13784 (toxin positive); 2, B. fragilis ATCC 43858 (toxin positive); 3, B. fragilis NCTC 9343 (type strain; toxin negative); 4, B. fragilis ATCC 23745 (toxin negative); 5 and 6, B. fragilis clinical isolates (toxin positive); 7 and 8, B. fragilis clinical isolates (toxin negative); 9, B. vulgatus (toxin negative); 10, B. distasonis (toxin negative); 11, B. thetaiotaomicron (toxin negative); lane M, DNA molecular mass marker (Marker V; Boehringer Mannheim). The 294-bp product, corresponding to the amplified portion of the enterotoxin gene, is indicated. The primers migrated to position p.

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TABLE 1. Results of the cytotoxicity test with HT-29 cells and PCR for 113 bacterial strains including B. fragilis, other Bacteroides spp., and some bacterial species known to produce enterotoxin and/or metalloprotease.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of isolates</th>
<th>Source</th>
<th>Test results</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. fragilis VPI 13784</td>
<td>1</td>
<td>Reference</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. fragilis ATCC 43858</td>
<td>1</td>
<td>Reference</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. fragilis NCTC 9343</td>
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<td>Reference</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>19</td>
<td>Feces</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>21</td>
<td>Clinical</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>19</td>
<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>16</td>
<td>Clinical</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>7</td>
<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
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<td>B. distasonis</td>
<td>6</td>
<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. thetaiotaomicron</td>
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<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. ovatus</td>
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<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
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<td>Feces</td>
<td>–</td>
<td>–</td>
</tr>
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<td>C. difficile</td>
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<td>Feces</td>
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<td>–</td>
</tr>
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<td>E. coli</td>
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<td>Feces</td>
<td>–</td>
<td>–</td>
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<td>V. cholerae</td>
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<td>Feces</td>
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<td>–</td>
</tr>
<tr>
<td>S. marcescens</td>
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<td>Clinical</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. aeruginosa</td>
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<td>L. pneumophila</td>
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<td>–</td>
<td>–</td>
</tr>
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</table>

* HT-29 cell cytotoxicity test.

obtained with $10^7$ CFU/g were very different for the samples from the two donors (Fig. 2). This suggests that some inhibitors were still present but were removed by dilution. The culture method was used with the double purpose of removing the inhibitors from feces and enriching the sample for B. fragilis. Amplification products were obtained from sweeps collected after 24 h of incubation with a sensitivity compared to that of the previous method, that is, $10^8$ to $10^7$ CFU/g of feces. By using sweeps from plates incubated for 48 h, the sensitivity was largely enhanced, so that products from $10^5$ to $10^4$ CFU/g of feces could be revealed (Fig. 3). There was clearly 1 log difference in the lower limit of detection between the samples from the two donors. This difference might reflect a diversity in the fecal flora of the two donors, possibly affecting the growth of ETBF on the plates. Fecal samples to which ETBF cells were not added were negative.

**PCR amplification of DNA from the feces of patients with diarrhea.** From the results obtained with the spiked stools, clinical fecal samples from children and adults with diarrhea were processed for PCR by the culture method only. Upon culture on BBE agar plates, the bacterial growth was less abundant and appeared later than the growth of the spiked samples. This might be due to the storage of the samples in the frozen state as well as to the dilution of microorganisms in loose or watery stools. Therefore, the sweeps were obtained only after 48 to 72 h of incubation. ETBF had been detected by conventional methods (culture, identification, and toxin detection) in 10 of the 18 samples examined. All except one of these samples gave a strong amplification product by PCR. One sample, which yielded scant colonies on the BBE agar plate, gave a faint, barely visible band. When the PCR assay was repeated with a more concentrated template (the 1:10 dilution of the sweep pellet was omitted), the amplification band was clearly visible in the gel (data not shown). Samples from which ETBF was not recovered by culture were consistently negative by PCR, regardless of whether other enteric pathogens were detected (Fig. 4).

**DISCUSSION**

The enterotoxin of B. fragilis has been the focus of several studies in the last few years, since this factor has been characterized to a molecular level and a tissue culture assay has been developed for its detection (9, 25, 27). Recent studies have suggested a role for ETBF in diarrheal diseases of children...
(17–19), although the high number of healthy carriers in some areas makes it difficult to define the pathogenic importance of this microorganism (16). The enterotoxin of \textit{B. fragilis} has been characterized as a metalloprotease (9), and similar to homologous enzymes in other bacteria, such as \textit{L. pneumophila} or \textit{P. aeruginosa} (5), it can represent a virulence factor for damaging the integrity and function of the intestinal mucosa. Therefore, more studies at both the epidemiological and the molecular levels are necessary to better understand the role of ETBF strains.

We developed a PCR assay to discriminate ETBF strains from nonenterotoxigenic strains of \textit{B. fragilis} and we compared the PCR results with the results of the cytotoxicity test with HT-29 cells, which is recognized to be both specific and sensitive for the detection of \textit{B. fragilis} enterotoxin (27). We found complete agreement between the biological test and the PCR, that is, between the enterotoxigenic phenotype and genotype for the strains examined. From our results, it follows that all ETBF strains possess the same or a very similar gene for the enterotoxin, while nontoxicogenic \textit{B. fragilis} strains lack the enterotoxin gene. The existence of strains possessing the enterotoxin gene and not expressing it cannot be ruled out, although this seems to be a rare event, at least among \textit{B. fragilis} isolates from humans. The other bacterial species investigated, including those belonging to the \textit{B. fragilis} group and those known to produce metalloproteases, neither produce a toxin active in the HT-29 cell assay nor are positive in the PCR. The primers chosen flank a segment in the gene that does not include the zinc-binding site of the enterotoxin, which contains a conserved motif identical to that in other metalloproteases (9). Therefore, the assay is very specific for the \textit{B. fragilis} enterotoxin.

Recently, the complete nucleotide sequences of the enterotoxin gene from two different ETBF strains, strain VPI 13784 and a strain of porcine origin, have been published by two independent groups of investigators (4, 6). In both cases, the enterotoxin gene was shown to code for a protein larger than the purified metalloprotease. The gene includes a signal peptide and a protease with a putative cleavage site for the release of the mature metalloprotease toxin. The genes from the two strains, although almost identical for most part of the protease, show reduced homology in the region coding for the mature protein. Therefore, it has been proposed that the enterotoxin gene isoforms from VPI 13784 and from the porcine strain be identified as \textit{bft}-1 and \textit{bft}-2, respectively (4). The PCR assay that we performed was able to amplify the expected portion of the enterotoxin gene from ETBF strains possessing either isoform (data not shown). The few mismatches between the sequences of the primers chosen and the published sequences of the genes (three mismatches in BF1 with both isoforms, and three mismatches in BF2 with isoform \textit{bft}-2 only) did not affect the results of the assay.

The establishment of ETBF as an enteric pathogen requires extensive epidemiological studies consisting of determination of the association between diarrhea and the presence of ETBF in stools in various settings. For such studies it is necessary to culture \textit{B. fragilis} from the feces, to isolate and identify this species among the complex fecal bacterial flora, and to test for enterotoxin production by the cell culture assay. These procedures require several days and the availability of facilities for anaerobic bacteriology and tissue culture. A direct PCR assay with stools can be a very useful alternative because of its rapidity and specificity.

It is well known that clinical samples such as blood, urine, and feces contain PCR inhibitors (30). Stools in particular can contain variable amounts of bilirubin and bile salts, which inhibit the activity of the thermostable DNA polymerase (29). A variety of methods have been devised to overcome this problem, although from the published literature it seems apparent that no single method works satisfactorily in two different laboratories or with two different PCR assays (21, 22). Moreover, some of the methods are very laborious, involving steps of spin washing and DNA extraction and purification that increase the danger of cross contamination, and therefore, they are not suitable for routine diagnostic procedures. A recently proposed method consists of enrichment of the bacterial species of interest from feces with the aid of immunomagnetic beads prior to PCR (29, 31). This technique is easy to perform and efficient; however, it requires the availability of monoclonal antibodies agglutinating the bacterial species of interest.

To test the feasibility of a PCR assay with feces, we reconstructed ETBF-containing fecal samples by adding known numbers of a reference ETBF strain to stool samples from two healthy volunteers. We chose to compare three methods which offered a simple means of sample preparation. The first method required only dilution of the specimen, digestion with proteases, and heating. Although this method was successfully used to detect \textit{C. difficile} (1) and Shiga toxin-producing \textit{E. coli} (8), it did not yield any amplification product by our assay. The samples had to be diluted 100-fold, because the reaction was inhibited when less dilute template was used, which was detrimental to the sensitivity of the assay.

The commercially available kit was very easy to use; the DNA is adsorbed onto the silica membrane, while the majority of the inhibitors are washed away. However, the samples needed 10-fold dilution prior to PCR because of the persistence of inhibitors, and the detection limit was 10⁵ CFU/g of feces.

The third method, already applied to the direct detection of a particular subpopulation of \textit{E. coli} from feces (20, 21), required cultivation of feces on a selective medium before the whole bacterial growth was collected (sweep plate) and used as a template for PCR. In this case, we have used a solid medium, BBE agar, as the most suitable medium for cultivating \textit{B. fragilis} from fecal samples (16); it contains gentamicin and bile and inhibits most of the facultative bacteria, while it enriches for \textit{Bacteroides} and allows for some differentiation among the \textit{B. fragilis} group (23). The method that we propose is simple and economical, because it requires that the whole culture on the plate be collected and processed without any attempt at species identification. The sensitivity obtained with the spiked samples (10⁶ to 10⁸ CFU/g of feces) was comparable to that obtained by other investigators in other PCR assays with feces (22, 29) and should be suitable for the detection of clinical infections, especially in consideration of the fact that if ETBF strains are present, they make up the vast majority of the \textit{B. fragilis} population in feces (16, 18).

In fact, when tested with a small number of clinical samples from patients with diarrhea, the sweep method proved to be sensitive and specific. For ETBF-positive fecal samples, the PCR product was in every case but one easily visualized by ethidium bromide staining. One positive sample gave a very faint band, possibly due to a loss of viability of \textit{B. fragilis} upon storage. In this case the use of a more concentrated DNA template in the assay was sufficient for obtaining a clearly positive result. PCR was consistently negative when it was performed with ETBF-negative fecal samples (diarrhea due to other enteropathogens of unknown cause). However, due to the limited number of clinical samples studied and to the fact that they had been stored frozen for various lengths of time, this method needs to be validated in larger, prospective studies.
REFERENCES


