Determination of bft Gene Subtypes in Bacteroides fragilis Clinical Isolates

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A rapid multiplex PCR approach was developed to detect the bft gene subtypes in Bacteroides fragilis clinical isolates. This technique could be used to look at the epidemiology of enterotoxigenic strains of B. fragilis in clinical infections and whether there is a correlation between disease and the presence of B. fragilis enterotoxin.

Bacteroides fragilis is the most commonly isolated anaerobe from human clinical infections and is considered to be the most virulent species in the B. fragilis group (6, 12). An association between enterotoxigenic strains of B. fragilis (ETBF) and diarrheal disease was first noted in livestock and was subsequently reported in human diarrheal disease in 1987 by Myers et al. (7–9). There is now growing evidence worldwide indicating that ETBF strains are associated with human diarrheal disease, with the majority of the studies conducted with young children (1, 10, 11). In addition, a study by Kato et al. implicated ETBF in approximately 30% of the bloodstream infections caused by B. fragilis (3). There are also several studies that have investigated the epidemiology and genotypic diversity of ETBF in human specimens (2, 3, 5). ETBF strains have been found to produce a 20-kDa zinc metalloprotease toxin, BFT (B. fragilis toxin), of which there are three distinct subtypes (14). In this study, a multiplex PCR was developed to investigate the presence of the three subtypes of B. fragilis enterotoxin (bft) gene in B. fragilis human clinical strains.

Three hundred twenty-nine B. fragilis strains were isolated from various clinical specimens (Table 1). All of these isolates originated from human clinical specimens and were identified and stored at the Wadsworth Anaerobe Laboratory, VA Medical Center, West Los Angeles, CA. B. fragilis VPI 13784 (produces BFT subtype 1), 86-5443-2-2 (produces BFT subtype 2), and Korea 419 (produces BFT subtype 3) were used as reference strains and were kindly donated by C. L. Sears of the Johns Hopkins University School of Medicine, Baltimore, MD. A nontoxigenic strain of B. fragilis, ATCC 25285, was used as a negative control. B. fragilis isolates were grown on brucella blood agar under anaerobic conditions at 37°C for 48 h.

The bft gene sequences of B. fragilis species retrieved from GenBank were analyzed by multisequence alignment with CLUSTAL-W (http://genome.kribb.re.kr). On the basis of the multisequence alignment analysis data, two reverse primers were newly designed (BFET-TYPE1, 5′-ATT GAA CCA GGA CAT CCC ‘3′ [specific for bft-1]; BFET-TYPE3, 5′-CGT GTG CCA TAA CCC CA-3′ [specific for bft-2]). A common forward primer, GBF-201 (5′-GAA CCT AAA ACG GTA TAT GT-3′), and a reverse primer, GBF-322 (specific for bft-2; 5′-CGC TCG GGC AAC TAT-3′), previously designed by Kato et al., were also used (4). The primer sequences were analyzed with the NetPrimer analysis software (http://www

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Sites related to the bowel</th>
<th>Sites related to lung</th>
<th>Sites related to female genital tract</th>
<th>Blood isolates</th>
<th>Skin and soft-tissue infections</th>
<th>Miscellaneous, unknown sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) of bft subtypes</td>
<td>no. (%) positive</td>
<td>no. (%) positive</td>
<td>no. (%) positive</td>
<td>no. (%) positive</td>
<td>no. (%) positive</td>
</tr>
<tr>
<td>Sites related to the bowel</td>
<td>BFT (B. fragilis toxin)</td>
<td>Appendix tissue</td>
<td>19 (12)</td>
<td>1 (2)</td>
<td>8 (14)</td>
<td>58</td>
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<tr>
<td></td>
<td>5′-ATT GAA CCA GGA CAT CCC ‘3′</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>8 (14)</td>
<td>2 (18)</td>
<td>1 (2)</td>
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<tr>
<td></td>
<td>5′-CGT GTG CCA TAA CCC CA-3′</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>8 (14)</td>
<td>2 (18)</td>
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<td>8 (14)</td>
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<td></td>
<td>BFET-TYPE3</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>8 (14)</td>
<td>2 (18)</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

Table 1. Detection of the bft subtypes in different clinical specimens

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The specificity of these primers was predicted by comparison to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) and were further tested by running PCR with DNA samples from 10 type strains of *B. fragilis* group species and 3 reference strains representing three subtypes of ETBF. To prepare a DNA sample for PCR amplification, a bacterial colony was taken from blood agar culture and resuspended in 200 μl of distilled water in a microcentrifuge tube. The sample was then boiled for 10 min and centrifuged for 1 min at 14,000 rpm, and 2 μl of supernatant was used for subsequent PCR amplification. PCR amplifications were performed in a 30-μl reaction mixture containing 1× buffer; 2.0 mM MgCl₂; 200 mM deoxynucleoside triphosphate; 20 pmol each of GBF-201, BFET-TYPE1, GBF-322, and BFET-TYPE3; 0.5 U of Taq DNA polymerase (Promega); and 2 μl of bacterial sample DNA. Conditions were optimized as follows: 5 min at 94°C; 3 min at 94°C, 1 min at 63°C, and 1 min at 72°C for 34 cycles; and 5 min at 72°C. Gel electrophoresis was performed for 45 min at 125 V on a 6% acrylamide gel, with visualization by ethidium bromide staining.

The developed multiplex PCR was able to detect the bft gene and distinguish among the three different subtypes. An amplification signal was detected from the three reference ETBF strains, which each carry a specific bft gene subtype but not from other reference strains tested (data not shown), showing that the PCR could specifically detect each bft subtype. An acrylamide gel showing the bands of amplified bft genes is shown in Fig. 1. The numbers and relative frequencies (expressed as percentages) of *B. fragilis* strains positive for the bft gene isolated from various clinical specimen types are shown in Table 1. The bft-1 subtype was the most prevalent (9%), followed by the bft-2 subtype (3%) and the bft-3 subtype (1%). Of the abscess isolates, 14 (18%) of 76 were found to have the bft gene, although this was found not to be statistically significantly different compared to nonabscess isolates. There were 49 blood isolates in this data set, and only 4 (8%) were found to have the bft gene. In total, the bft gene was found in 13% of the *B. fragilis* isolates from our clinical specimens.

There are various conflicting reports about ETBF and human clinical disease. Some studies have shown that ETBF is present in the normal colonic flora of humans, as well as in clinical infections (1, 10). Other studies have found a significant number of ETBF isolates among certain *B. fragilis* clinical isolates, suggesting their importance as a virulence factor in these infections. For example, Kato et al. found that 28.1% of the *B. fragilis* blood isolates that they tested were ETBF and this was statistically significantly different compared to non-blood isolates (of which 13.7% were ETBF) (3). They also found that bft-1 was the predominant subtype. Our study found bft-1 to be the predominant subtype, but unlike Kato et al., we did not find a significantly high number of blood isolates with the bft gene. Our study also found three isolates with the bft-3 gene, whereas other studies have found no bft-3 subtypes at all (13). These differences may be attributed not only to the types of clinical specimens studied but also to patient demographics (studies have been conducted in various countries with different geographical and social populations) and even sample size. Further studies are needed to understand why there is diversity in the presence of the bft subtypes and the possible significance of ETBF in clinical infections (12). The multiplex PCR approach that we describe provides a convenient, dependable, and rapid method for these studies, which require enough patients in the various clinical categories to determine statistically significant differences and to better understand the epidemiology of *B. fragilis* diarrheal disease and its relationship to bft as a virulence factor.

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REFERENCES


FIG. 1. Multiplex PCR for detecting the bft gene subtypes in *B. fragilis*. Lanes: 1, 50-bp DNA molecular size ladder (Promega); 2 and 6, *B. fragilis* 1350 and VPI 13784 (bft-1); 3 and 7, *B. fragilis* 1337 and 86-5443-2-2 (bft-2); 4 and 8, *B. fragilis* 9075 and Korea 419 (bft-3); 5, nonenterotoxigenic *B. fragilis* 1360.

