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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 T-3′ [specific for *bft-1*]; BFET-TYPE3, 5′-CGT GTG CCA TAA CCC CA-3′ [specific for *bft-3*]). 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 1. Detection of the *bft* subtypes in *B. fragilis* isolates from different clinical specimens

Specimens	No. of strains tested	No. (%) of *bft* subtype:			Total no. (%) positive
		bft-1	*bft-2*	*bft-3*	
Sites related to the bowel					
Appendix tissue	58	7 (12)	0	1 (2)	8 (14)
Peritoneal fluid	51	2 (4)	2 (4)	0	4 (8)
Intra-abdominal abscess	41	7 (17)	1 (2)	0	8 (19)
Sacral abscess	3	1 (33)	0	0	1 (33)
Liver abscess	4	0	0	0	0
Other abscesses	10	2 (20)	1 (10)	0	3 (30)
Bile	3	1 (33)	0	0	1 (33)
Stool	6	0	0	0	0
Sites related to lung					
Pleural fluid	11	2 (18)	0	1 (9)	3 (27)
Transtracheal aspirate	2	1 (50)	0	0	1 (50)
Lung tissue	2	0	0	0	0
Sites related to female genital tract					
Cervix	2	0	0	0	0
Lochia	1	0	0	0	0
Blood isolates					
Blood	49	2 (4)	1 (2)	1 (2)	4 (8)
Catheter tip, intravenous	1	0	0	0	0
Skin and soft-tissue infections					
Abscesses	18	2 (11)	0	0	2 (11)
Ulcers	4	0	2 (50)	0	2 (50)
Wounds	15	0	1 (7)	0	1 (7)
Miscellaneous, unknown sites	48	3 (6)	3 (6)	0	6 (12)
Total	329	30 (9)	11 (3)	3 (1)	44 (13)

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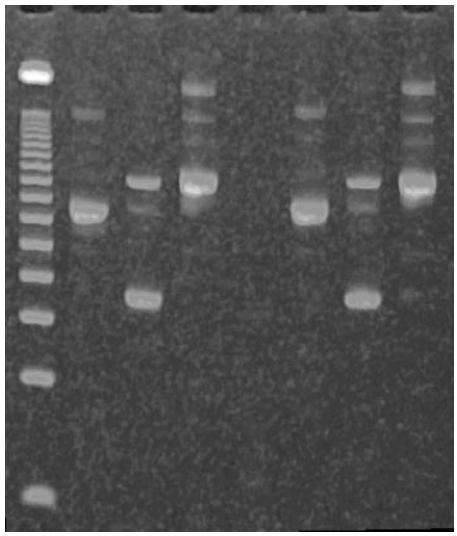

FIG. 1. Multiplex PCR for detecting the *bft* gene subtypes in *B. fragilis*. Lanes: 1, 50-bp DNA molecular size ladder (Promega); 2 and 6, *B. fragilis* 1350 and VPI 13784 (*bft-1*); 3 and 7, *B. fragilis* 1337 and 86-5443-2-2 (*bft-2*); 4 and 8, *B. fragilis* 9075 and Korea 419 (*bft-3*); 5, nonenterotoxigenic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

.premierbiosoft.com/netprimer). The specificities of these primers were 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 $MgCl_2$; 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 nonblood 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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