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Quantitative Detection of Enterotoxigenic *Bacteroides fragilis* Subtypes Isolated from Children with and without Diarrhea▽

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A rapid real-time PCR (RT-PCR) approach was developed to detect the *bft* gene subtypes in *Bacteroides fragilis* isolated from fecal samples. DNA obtained from diarrhea (110) and nondiarrhea (150) samples was evaluated. Subtype 1 was observed in 9 (8.2%) diarrhea and 7 (4.7%) nondiarrhea samples. Subtype 2 was not detected in any DNA samples, and subtype 3 was observed in only 1 diarrhea sample. The presence of the *bft-1* gene did not show any statistically significant differences between the groups of children. This technique could be used to evaluate a possible correlation between disease and the presence of *B. fragilis* enterotoxin.

Bacteroides fragilis is considered to be the anaerobe most commonly isolated from human clinical infections and is also considered to be the most virulent species in the *Bacteroides fragilis* group (11).

Enterotoxigenic *B. fragilis* (ETBF) strains have been associated with diarrhea in young farm animals and humans (2, 9). The enterotoxigenic strains were also isolated at higher frequencies from patients with bacteremia and infections of different sites related to the lung, intestine, abscesses, and blood (1). However, several clinical studies demonstrated that 5 to 20% of the population in the world appear to carry these strains asymptomatically (5, 10).

Several studies have shown an association between ETBF strains and human diarrheal disease, particularly in young children (7, 8, 13). ETBF strains produce a 20-kDa zinc metalloprotease toxin, BFT (*B. fragilis* toxin), of which there are three distinct subtypes (14). These enterotoxigenic strains produce different isotypes of the enterotoxin, which are associated with the *bft-1*, *bft-2*, and *bft-3* genes (4, 6). These isotypes display different potencies, as follows: BFT-2 > BFT-1 > BFT-3 (12, 14). In this study, a real-time PCR (RT-PCR) was developed to investigate the presence of these subtypes of the *B. fragilis* enterotoxin (*bft*) gene in stool samples.

DNA from fecal samples obtained from 110 children with diarrhea (54 boys and 56 girls) and from 150 children without diarrhea (71 boys and 79 girls) was obtained; all children were 1 month to 10 years old. None of the children was under antibiotic treatment for at least 3 months prior to sample collections. The Ethics Committee of the Instituto de Ciências Biomédicas at the University of Sao Paulo (process no. 743/CEP) approved this study. A nontoxigenic strain of *B. fragilis*, ATCC 25285, was used as a negative control.

The *bft* sequence of *B. fragilis* retrieved from GenBank was analyzed by multisequence alignment with CLUSTAL W. On the basis of the multisequence alignment analysis data, six primer pairs and three probes were designed (Table 1). Primers and probes were also analyzed with NetPrimer analysis software (http://www.premierbiosoft.com/netprimer). The specificities of the primers and probes were predicted by comparison to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST), and primers were further tested by running the PCR with DNA samples from 10 type strains of *B. fragilis* group species and 3 reference strains representing three subtypes of ETBF. The DNA samples for PCR were obtained from approximately 200 mg of fresh stools by using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) and stored at −80°C until use.

In order to detect the *bft* gene, a qualitative PCR was performed by using specific primers, according to the study by Pantosti et al. (9), and in order to detect the *bft* gene subtypes, a multiplex PCR was performed by using specific primers, according to the study by Kato et al. (6). Real-time PCR assays were performed using a Rotor Gene 6000 (Corbett Life Science, Mortlake, New South Wales, Australia). All assays were carried out in duplicate and performed in a total volume of 25 μl, containing 2× TaqMan universal master mix (Applied Biosystems, Foster City, CA), 100 μM each primer, 100 μM TaqMan probe, and 2 ng DNA. Amplifications were performed in a thermocycler programmed as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 58°C for 1 min. The negative control used was a PCR TaqMan master mix without DNA. The primer/probe sets used are shown in Table 1. The standard curve was analyzed for each evaluated *bft* gene using the respective primer/probe set against a serial dilution of each bacterial DNA sample obtained from *B. fragilis* GAI 97124, *B. fragilis* VPI 13784 (*bft-1*), *B. fragilis* 86-5443-2-2 (*bft-2*), and *B. fragilis* Korea (*bft-3*), corresponding to 10^{-2} to 10^{-7} CFU per g of feces. DNA samples were obtained by using an Easy-DNA kit (Invitrogen Corp., Carlsbad, CA). Amplifications were adjusted using R^2 values of >0.900. A sample was considered positive for a target gene when the fluorescence emitted by the sample was at least 50%

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TABLE 1. Target genes and sequences of primer and probe sets used in real-time PCR[a]

Gene	Primer sequence (5′→3′)
bft (ETBF)	F-GGG ACA AGG ATT CTA CCA GCT TTA TA
	R-ATT CGG CAA TCT CAT TCA TCA TT
	P-CGC AAT GGC GAA TCC ATC AGC TAC A
bft-1 subtype	F-GGG ATG TCC TGG TTC A
	R-AAT TAT CCG TAT GCT CAG CG
	P-CTT CGG ATT TTR AAG CCA GTG GGA TGT C
bft-2 subtype	F-CTT AGG CAT ATC TTG GCT TG
	R-GCG ATT CTA TAC ATG TTC TC
	P-CTT CGG ATT TTR AAG CCA GTG GGA TGT C
bft-3 subtype	F-TTT GGG CAT ATC TTG GCT CA
	R-ATC ATC CGC ATG GTT AGC A
	P-CTT CGG ATT TTR AAG CCA GTG GGA TGT C

[a] The annealing temperature used for all genes was 58°C, and all data were obtained in this study. F, forward primer; R, reverse primer; P, probe.

TABLE 2. Quantitative analysis of the different *bft* genes among ETBF strains from diarrhea and nondiarrhea fecal samples

Target gene	No. of positive samples (range of $\log_{10}$ no. of copies [mean ±SD])		*P* value
	Diarrhea	Nondiarrhea	
bft	10 (2.4 to 7.5 [4.5 ± 1.8])	7 (2.2 to 6.1 [3.9 ± 1.3])	0.46
bft-1	9 (1.1 to 7.3 [3.7 ± 1.8])	7 (2.2 to 5.6 [3.8 ± 1.2])	0.91
bft-3	1 (0.34)	0	

higher than the limit of detection previously established for the *bft* gene and 50% above the background fluorescence.

Mean values ± standard deviations (SD) were calculated for each bacterial subtype, and the Student *t* test was used. A $\log_{10}$ value for the copy number for each gene in each fecal sample was computed. A difference of P of <0.05 was considered statistically significant.

By using a conventional PCR and a multiplex PCR, DNA samples were positive for the *bft* genes. The developed RT-PCR was able to detect the target copy number and distinguish among the three different subtypes. An amplification signal was detected from the target genes in the reference strains, which each carry a specific *bft* gene subtype, but was not detected in other reference strains tested (data not shown), showing that the PCR could specifically detect each *bft* subtype.

The numbers of positive samples and the target copy numbers of the *bft* genes are shown in Table 2. The *bft* gene was observed in 10 (9.1%) of 110 diarrhea samples, with 9 of them subtyped as *bft-1* and 1 subtyped as *bft-3*. In addition, the *bft* gene was observed in 7 (4.7%) of 150 nondiarrhea samples, and all of them were subtyped as *bft-1*. No DNA sample harbored the subtype *bft-2*. The quantitative analysis of DNA obtained from diarrhea samples showed copy number values that ranged from $\log_{10}$ 2.4 to $\log_{10}$ 7.5 for the *bft* gene, from $\log_{10}$ 1.1 to $\log_{10}$ 7.3 for the *bft-1* gene, and of $\log_{10}$ 0.34 for the *bft-3* gene. For DNA obtained from nondiarrhea samples, the copy number values ranged from $\log_{10}$ 2.2 to $\log_{10}$ 6.1 for the *bft* gene and from $\log_{10}$ 2.2 to $\log_{10}$ 5.6 for the *bft-1* gene. In all PCR amplifications, gene copy numbers ranged from $\log_{10}$ 1 to $\log_{10}$ 7. By using the specific primer/probe sets described here, detection limits of 12 copies for the *bft-1* gene and 2 copies for the *bft-3* gene were observed. There are various conflicting reports about ETBF and human clinical disease. Some studies have shown that ETBF is present in the normal colonic microbiota of humans as well as in clinical infections (3, 11). Studies have shown *bft-1* to be the predominant subtype, and to our knowledge, this is the first report showing a quantitative RT-PCR used for *bft* gene subtyping.

Some studies have been performed in Brazil that detected the *bft* gene in fecal DNA obtained from children with acute diarrhea, but this gene was not observed in DNA samples obtained from normal feces (3, 7). Ulger Toprak et al. (13) described the detection of *bft-1* and *bft-2* in the feces of colon cancer patients, normal feces, and extraintestinal strains, and the *bft-1* gene was more commonly detected than *bft-2*. In contrast, in this study, only one *bft-3* gene and no *bft-2* genes were detected. It was a real surprise because in different reports, the *bft-2* gene was shown to be more common than the *bft-3* gene.

The quantitative RT-PCR approach that we described provides a convenient, dependable, and rapid method to study the diversity of the presence of the *bft* subtypes and the significance of ETBF in clinical infections (1). Considering the gene copy numbers determined for each sample, no statistically significant value among both child groups was observed. However, it is also important to determine statistically significant differences and to better understand the true role of ETBF strains and *bft* gene subtypes as virulence factors. Additionally, since differences in detecting the three *bft* gene subtypes may be attributed not only to the types of clinical specimens but also to patient demographics, these results indicate the need for more studies to evaluate the role of each subtype in the pathogenesis of diarrhea in different hosts.

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