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bft gene subtyping in enterotoxigenic *Bacteroides fragilis* isolated from children with acute diarrhea

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Abstract

Enterotoxigenic *Bacteroides fragilis* (ETBF) strains are associated with diarrhea disease in farm animals and young children. In this study, the *bft* gene subtyping from ETBF strains recovered from one immunodeficient and two immunocompetent children with diarrhea were determined. Thirteen ETBF strains were isolated and by using a multiplex-PCR their *bft* subtypes were determined. All 13 ETBF strains harbored the *bft*-1 subtype and by AP-PCR they were clustered in the same group I. This study shows that ETBF strains can be present in acute diarrhea and that *bft*-1 subtype is often present in these organisms. However, further studies are needed to evaluate the role of this *bft*-1 subtype in the pathogenesis of diarrhea.
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Keywords: Enterotoxigenic *Bacteroides fragilis*; *bft* gene subtype; Diarrhea

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1. Introduction

Enterotoxigenic *Bacteroides fragilis* (ETBF) strains have been associated with acute diarrhea in farm animals and young children, and they produce a zinc-dependent metalloprotease enterotoxin (BFT) that stimulates a fluid accumulation in lamb ligated ileal loops and that generates reversible morphological changes of the HT29/C_1 cells [1–5]. This enterotoxin is called fragilysin and it is a metalloprotease of 44.4 kDa, containing a signal peptide of 18 amino acids; by intracellular processing it results in an active mature toxin of 20.7 kDa (186 amino acid residues) [6].

Studies have shown the presence of ETBF in 6–12% of children with diarrhea in USA, 11% in Italy, 12% in Sweden, 5–15% in Japan and 2–6% in Bangladesh [3,7–10]. Studies show that subjects with or without diarrhea can harbor ETBF organisms, and they may be present in small numbers in the human intestinal indigenous microbiota [11,12].

Immunocompromised patients, particularly patients with AIDS are more susceptible to several infections, including enteric infections and anaerobic infections by several organisms such *Bacteroides* spp., *Clostridium* spp., *Clostridium difficile*, *Fusobacterium* spp., *Prevotella* spp., *Peptostreptococcus* spp., *Escherichia coli, Shigella flexneri, Campylobacter* spp., *Cryptosporidium* sp., *Microsporidium* sp., *Isospora belli, Giardia intestinalis, Entamoeba histolytica, Strongyloides stercoralis* and others, due to disruption of normal mucosal or cutaneous barriers, prolonged antibiotic use, and hospitalization [13–18].

ETBF strains contain an exclusive 6-kb region harboring both the *bft* and *mp*II genes termed of *B. fragilis* pathogenicity island (BfPAI) plus a flanking DNA region of 18-kb in size, which appear to be essential to the expression of high levels of the biologically active BFT [19]. Moreover, three *bft* alleles were firstly identified in the 6-kb region, then cloned and sequenced. The identity of the nucletotide and amino acid sequences, respectively, is

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95.3% and 93.2% between *bft*-1 and *bft*-2, 95.1% and 94% between *bft*-1 and *bft*-3, and 97.1% and 96.7% between *bft*-2 and *bft*-3 [20]. The *bft*-1 gene was found in the VPI 13784 strain in isolated from lamb, while *bft*-2 and *bft*-3 genes were found in 86-5443-2-2 and in Korea 419 strains, isolated from piglet and from a human subject, respectively [19–21].

ETBF can produce different isotypes of the enterotoxin the most common being these encoded by the *bft*-1 and *bft*-2 genes [19]. Although these isoforms display the same biological activities, and it has been suggested that their potencies might be different [22]. BFT biologic activity has been detected on both renal and intestinal epithelial cell lines, e.g. HT-29/C1, Caco-2, T84, MDCK, HCT-8, capable of forming tight junctions, but not on epithelial cell lines, such as HeLa or Hep-2 lacking this property. In vitro this activity has shown that BFT-2 exhibits subtle greater biologic activity than BFT-1 whereas BFT-3 may be less active than either BFT-1 or BFT-2 [23]. Moreover, gnotobiotic mice monoassociated with *bft*-2 subtype showed lesions slightly more severe than *bft*-1 subtype [24].

Studies have shown that *bft*-1 is the predominant subtype in ETBF strains isolated from adults with diarrhea, while *bft*-2 is often found in strains isolated from children with antibiotic-associated diarrhea, as well as in cows, but further studies of the epidemiology and the clinical impact of ETBF are necessary [20,21].

Several methods for detecting phenotypic or genotypic variation have been used to characterize bacterial isolates and to distinguish strains that are potential pathogens from commensal strains. Genotypic methods, such as rRNA restriction fingerprint length polymorphism (RFLP), restriction endonuclease analysis (REA), arbitrarily primed-PCR (AP-PCR) and ribotyping were also used to analyse *B. fragilis* strains, and a high genetic diversity has been found [25]. In this study, the *bft* gene subtypes in ETBF strains isolated from AIDS and healthy children with diarrhea were determined.

2. Materials and methods

Thirteen ETBF strains were isolated from stool samples of three children as follow: six strains from one AIDS child and seven strains from two immunocompetent children with acute diarrhea, from March to October 2001 [26], and they were stored at −80 °C at the Anaerobe Laboratory of the University of São Paulo. Selected children were visiting two Brazilian hospitals (Instituto de Infectologia Emílio Ribas and Instituto da Criança do Hospital das Clínicas, São Paulo, SP, Brazil). The Ethic Commission of the hospitals and of the Instituto de Ciências Biomédicas, USP (Proc. 158/CEP) approved this study. The strains were subcultured in BHI broth in anaerobiosis, for 48 h, and they were submitted to a new identification by using a Rapid ID 32A kit (bioMérieux). Moreover, supernatants were used in the cytotoxicity assay of HT/$^{29}C_1$ cells and they were considered to contain fragilysin when a cytotoxic effect was visible after 4 h of incubation [26]. An enterotoxin-positive strain *B. fragilis* GAI 97124 was used as control.

In order to detect the *bft* gene, one colony of each isolate grown in blood agar was suspended in 300 µl of Milli-Q ultra-pure water, homogenized, boiled for 20 min and centrifuged at 14 000*g*, for 10 min. Supernatants were used as templates in PCR reaction performed with specific primers, according to Pantosti et al. [12]. Amplifications were performed in final volumes of 25 µl containing 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP (Invitrogen), 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), 0.4 µM primers and 10 ng DNA. Thermocycler (Perkin Elmer Amp PCR System 2400) was programmed to: 1 cycle of 94 °C (5 min), followed by 35 cycles of 94 °C (1 min), 52 °C (1 min), 72 °C (1 min) and 1 cycle of 72 °C (5 min). The amplified products were analysed in 1% agarose gel stained with ethidium bromide (0.5 µg/ml) and photographed by using a Kodak Digital Science System DC-120. The PCR amplification generated a 294 bp amplicon.

The *bft* gene subtypes were determined by using a multiplex-PCR, according to Kato et al. [20]. The bacterial DNA was obtained with the Easy-DNA kit (Invitrogen) and used as template. The reaction was carried out in a final volume of 25 µl containing 0.5 U Platinun *Taq* polymerase, 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP, 0.4 µM primers and 10 ng DNA. Thermocycler was programmed to: 1 cycle of 94 °C (5 min), followed by 35 cycles of 94 °C (30 s), 62 °C (1 min) and 72 °C (2 min) and 1 cycle of 72 °C (5 min). The amplified products were analysed in 1% agarose gel and stained with ethidium bromide. The expected amplicons were 190 bp for *bft*-1, 175 bp for *bft*-2 and 287 bp for *bft*-3.

The PCR amplified fragments encompassing the *bft* genes subtypes were extracted and purified by using a commercial kit (Concert Rapid Gel Extraction System, Invitrogen). PCR amplification was performed in a thermocycler (PT-100, MJ Research) with 35 cycles of 94 °C (20 s) and 62 °C (2 min) for both annealing and extension steps. Then, the amplified PCR products were purified with a DNA extraction kit (QIAquick Gel Extraction Kit, Qiagen), and the nucleotide sequence was determined by using a dye terminator cycle sequencing ready reaction kit (PE Applied Biosystem) for ABI prism 310 automated DNA sequencer (PE Applied Biosystem), according to the manufacturer's instructions.

The sequence obtained was assembled, edited and analysed with sequences of the *bft* gene retrieved from the NCBI databank (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were carried out with the Clustal analysis program (http://www.dbbm.fiocruz.br) and the assembling of the *bft* sequences were carried out with a Dnastar software version 4.

Moreover, the amplified fragments of each subtype were used as probes. They were purified by using the Concert™ Rapid Gel Extraction System kit (Invitrogen) and labeled by nick translation with [α-^{32}P] dCTP using the

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Ready-to-go DNA Labeling Beads (Amersham Biosciences, UK, Ltd). The probes were purified with the Probe Quant G-50 Micro Columns (Amersham) and used in colony hybridization assays [27] under high-stringency conditions, as follows: 5 × SSC (1 × SSC is 3 M NaCl, plus 0.3 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 2 × Denhardt's solution (Denhardt′s Solution 100 × Eppeendorf, Hamburg, Germany) and 0.1 mg/ml of sheared salmon sperm DNA (Eppendorf), at 65 °C, for 18 h. Washes were conducted at 65 °C, for 20 min with 0.1 × SSC and 0.1% SDS (twice), and 0.05 × SSC plus 0.1% SDS (twice). Filters were dried and exposed to X-ray films for 18 h, according to the manufacturers' instructions.

In order to evaluate if all the 13 strains belong to the same clone, a genotyping analysis was performed by using an AP-PCR with five decamer oligonucleotides of random sequences (GC content ranging from 60% to 100% and Tm ranging from 34 to 36 °C) in according to Eribe and Olsen [28]. Only primer OPA-3 (5′-AGT CAG CCA C-3′) generated a few distinct bands and showed at least two polymorphic DNA patterns among isolates tested. Amplifications were carried out in a final volume of 25 μl and thermocycler was programmed as described above but using an annealing temperature of 36 °C. For all strains, the AP-PCR band differences were scored visually. A combination of scores was then coded in a binary matrix. Pairwise similarities were computed by the program package NTSYS (Applied Biostatistic, inc. version 1.7) using SM coefficient of similarity and unweighted pair group method using arithmetic averages (UP-GMA) clustering. The enterotoxin-positive strains *B. fragilis* GAI 97124 and *B. fragilis* ATCC 43858, *A. viscosus* ATCC 91014 and *E. coli* J53 (pACYC 184) were used as controls.

3. Results

All the 13 ETBF strains showed the capacity of altering the HT-29/C_1 cells and by using a PCR reaction amplified a 294-bp fragment, as also showed by Bressane et al. [26]. The multiplex-PCR of the 13 ETBF isolates DNA generated amplicons with the predicted size of 190-bp for *bft*-1 (data not shown) and the *B. fragilis* GAI 97124 and *B. fragilis* ATCC 43858 used as controls belonged to the *bft*-2 subtype. Both 190 and 175 bp amplicons were sequenced and they were identical to the previously reported nucleotide sequences to subtypes *bft*-1 and *bft*-2 (Fig. 1), respectively. Moreover, by using high stringent conditions in the colony blot assay, all *bft* gene positive strains hybridized with the *bft*-1 probe. The AP-PCR method was employed and the amplifications with OPA-03 primer resulted in clear and reproducible DNA patterns for all the analysed isolates (Fig. 2). Strains showed amplicons from 0.55 to 2.9 kb, and three groups were observed. All the 13 strains were clustered with 86% of similarity in group I. It is important to mention that these 13 strains were isolated from one AIDS child (I) and two immunocompetent children (P), all of them with acute diarrhea. Moreover, strains I2 and I3, P21 and P22, and P19 and P20, respectively, showed 100% of similarity. Strain isolated from AIDS child showed high similarity (>0.85%) with the strains from immunocompetent children. Reference strains *B. fragilis* GAI 97124, *B. fragilis* ATCC 43858 and *A. viscosus* ATCC 91014 belonged to group II and *E. coli* J53 (pACYC 184) to group III (Fig. 2).

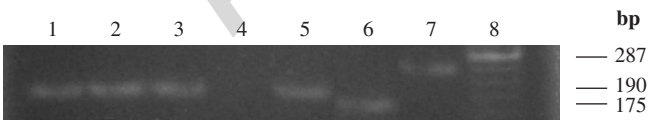

Fig. 1. *bft* gene subtyping by multiplex-PCR. Lanes 1–3, *bft*-1 subtypes (*B. fragilis* P13c, *B. fragilis* I15b, *B. fragilis* I15c); lane 4, non-enterotoxigenic *B. fragilis* S45a; lane 5, *B. fragilis* VPI 13784 (*bft-1*); lane 6, *B. fragilis* 86-5443-2-2 (*bft-2*); lane 7, *B. fragilis* Korea 419 (*bft-3*); lane 8, molecular weight 50 bp DNA ladder (Gene Ruler™).

4. Discussion

According to the World Health Organization diarrhea is among the leading causes of mortality of children worldwide, and etiologies of up to 50% of diarrhea illness causes remain unknown [29]. In this study, we evaluated the presence of the *bft* gene subtypes among 13 ETBF strains recovered from AIDS and non-AIDS children with acute diarrhea. All the 13 ETBF strains were subtyped as *bft*-1 and the sequence of each gene subtype was identical to those described at the Genbank.

The multiplex PCR used here proved to be a reliable and an effective technique for identifying subtypes of the *bft* gene from enterotoxigenic *B. fragilis*. Moreover, the *bft*-1 gene was the subtype only found among the examined strains, in accordance with Kato et al. [20], and the identity between the nucleotide sequences was 95.3% for *bft*-1 and the predictive amino acid sequences was 93.2% between *bft*-1 and *bft*-2. The conditions used in the hybridization assays were efficient in discriminating the *bft*-1, but not among *bft*-1 and *bft*-2 alleles due to the high similarity between the nucleotide sequences.

The lack of *bft*-2 and *bft*-3 in our strains might be due to different properties of our geographic area, dietary habits, genetic background or even immunologic deficiencies of our evaluated patients. Moreover, no relationship among clonal origin and clinical data of diarrhea in AIDS patients was observed.

In our study, it was possible to verify the discriminatory power of AP-PCR method using the OPA-03 primer among the 13 ETBF strains. The genetic diversity in *B. fragilis* group species using different molecular approaches has been evaluated but not always a clear genetic distance among isolates could be verified [30,31]. In our study, all the tested strains were clustered with 86% of similarity in group I. ETBF strains isolated from one AIDS child (strains I2 and I3) and immunocompetent child (strains P21 and P22, and P19 and P20), respectively, belonged to the same clone (Fig. 2). The reference strains *B. fragils* GAI

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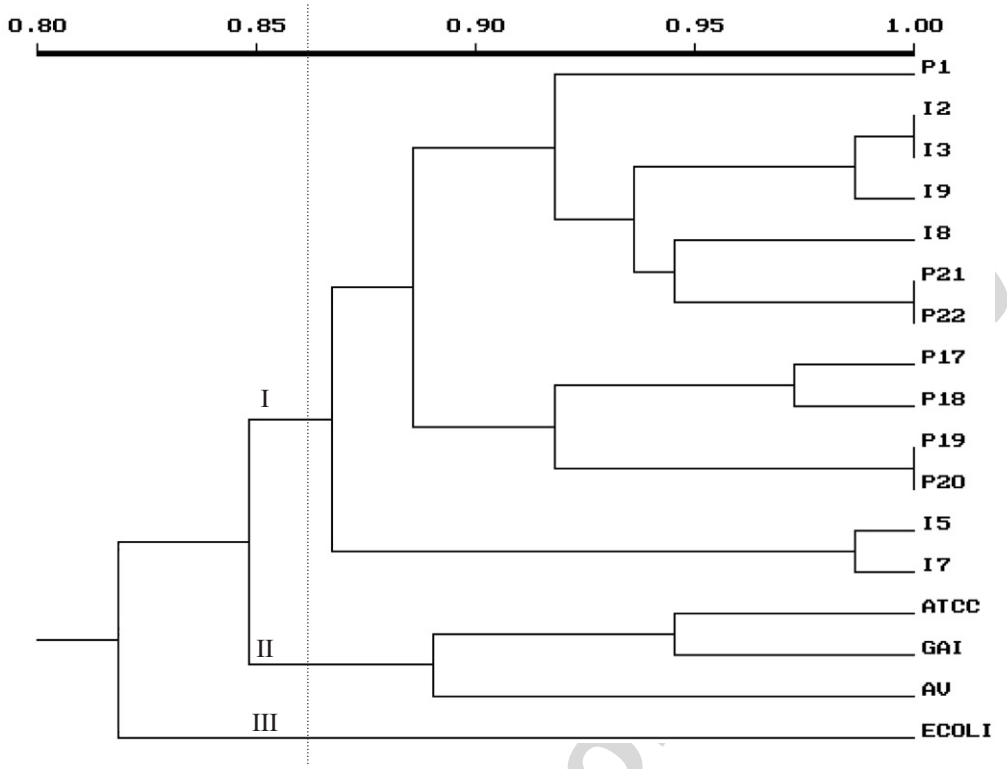

Fig. 2. Genotyping analysis of the 13-enterotoxigenic *B. fragilis* isolated from children with acute diarrhea. I: AIDS-child with diarrhea; P: immunocompetent children with diarrhea; ATCC: *B. fragilis* ATCC 43858, GAI: *B. fragilis* GAI 97124, AV: *A. viscosus* ATCC 91014, Ecoli: *E. coli* J53 (pACYC 184).

97124, *B. fragilis* ATCC 43858, *A. viscosus* ATCC 91014 and *E. coli* J53 (pACYC 184) were clustered in groups II and III, respectively. These results show the heterogeneity and the non-clonal origin of the ETBF strains, particularly those isolated from AIDS patients, in according to Smith and Callingham [32] and Martirosian et al. [33]. The existence of bacterial clones in the intestinal microbiota is not only restricted to *Bacteroides* species, Studies have showed that clonal origin in other anaerobes, such as *Fusobacterium nucleatum* [34] and *Porphyromonas gingivalis* [35].

Finally, it would be of interest to determine whether variation in the *bft* gene affects the pathogenicity in ETBF strains, and it may be worthwhile to study in vitro and in vivo the virulence of each subtype. Additionally, these results indicate the need of more studies to evaluate the role of each subtype in the diarrhea pathogenesis in different hosts.

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