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Research Paper

Enterotoxigenic and non-enterotoxigenic *Bacteroides fragilis* from fecal microbiota of children

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Abstract

Enterotoxigenic *Bacteroides fragilis* (ETBF) is an important part of the human and animal intestinal microbiota and is commonly associated with diarrhea. ETBF strains produce an enterotoxin encoded by the *bft* gene located in the *B. fragilis* pathogenicity island (BfPAI). Non-enterotoxigenic *B. fragilis* (NTBF) strains lack the BfPAI and usually show two different genetic patterns, II and III, based on the absence or presence of a BfPAI-flanking region, respectively. The incidence of ETBF and NTBF strains in fecal samples isolated from children without acute diarrhea or any other intestinal disorders was determined. All 84 fecal samples evaluated were *B. fragilis*-positive by PCR, four of them harbored the *bft* gene, 27 contained the NTBF pattern III DNA sequence, and 52 were considered to be NTBF pattern II samples. One sample was positive for both ETBF and NTBF pattern III DNA sequences. All 19 *B. fragilis* strains isolated by the culture method were *bft*-negative, 9 belonged to pattern III and 10 to pattern II. We present an updated overview of the ETBF and NTBF incidence in the fecal microbiota of children from Sao Paulo City, Brazil.

Key words: *Bacteroides fragilis*, children, ETBF, NTBF.

INTRODUCTION

The intestinal tract is a complex ecosystem and the relationships between the microbiota and the host can result in symbiotic and/or pathogenic outcomes (Hooper and Gordon, 2001). The colon is known to contain up to 10^{13} bacteria/g of feces and the bacterial community predominantly belong to the *Firmicutes* and *Bacteroidetes* phyla. *Bacteroides* species comprise about 30% of the total cultivable microbiota, and among them *Bacteroides fragilis* is an important part of the human and animal microbiota and one of the most studied intestinal species (Karlsoon *et al.*, 2011; Wick and Sears, 2010).

This microorganism has been associated with extra-intestinal infections, such as intra-abdominal, lung and brain abscesses (Tzianabos *et al.*, 1994). Some strains are able to produce an enterotoxin (fragilysin or BFT) which is encoded by the *bft* gene located in a 6-kb pathogenicity island (BfPAI). These strains are called enterotoxigenic *B. fragilis* (ETBF, pattern I) strains and are associated with acute diarrhea in humans and animals. Non-enterotoxigenic *B. fragilis* (NTBF) strains are also found in intestines of healthy individuals. They lack the BfPAI and represent two genetic patterns, pattern II (without a 12-kb BfPAI flanking region) and pattern III (with the flanking region) (Franco *et al.*, 1999).

Studies in Bangladesh, Turkey and Vietnam showed a consistent association of ETBF with human diarrhea in children under five years of age (Durmaz *et al.*, 2005; Nguyen *et al.*, 2005; Sack *et al.*, 1994). Pantosti *et al.* (1997) reported a high rate of ETBF carriage among healthy subjects, suggesting that ETBF rates in the human intestinal microbiota may vary widely depending on geographical areas.

Diarrhea is one of the most common diseases in humans and is considered a serious risk to infants in developing countries. In Brazil, the ETBF rate is low ranging from 1.5 to 3% (Antunes *et al.*, 2004; Bressane *et al.*, 2001;

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Kryzanowsky *et al.*, 2003). However, Miranda *et al.* (2008) showed an increase in the incidence of ETBF and NTBF, particularly NTBF pattern III strains. Merino *et al.* (2011) confirmed the presence of ETBF in children without diarrhea, suggesting that children could be a reservoir for these strains however, the presence of NTBF patterns II and III was not evaluated.

The presence of BfPAI flanking region observed in NTBF pattern III strains may indicate that the toxin gene can be transferred horizontally from ETBF to NTBFstrains (Franco *et al.*, 1999). This shows the need of evaluating the distribution of ETBF and NTBF strains in the intestinal microbiota of healthy children as eventual reservoirs of these microorganisms. In this study, the distribution and prevalence of *B. fragilis*, as well as the incidence of ETBF and NTBF strains in healthy children, were determined.

Materials and Methods

Samples

Normal fecal samples were collected from 84 children (46 boys and 38 girls) between the ages of 3 and 12 years old who had no signs of acute diarrhea or other intestinal disorders. The children were private and municipal schools students from Sao Paulo City, SP, Brazil. No child had undergone antibiotic therapy in at least 3 months prior to the sample collection. This study was approved by the Ethic Committee of the Institute of Biomedical Sciences of the University of Sao Paulo (No. 1043/CEP). The fecal samples were directly plated onto Bacteroides Bile Esculin (BBE) agar medium and incubated in anaerobiosis at 37 °C for 4 days. Characteristic colonies from each plate were subcultured on Brain Heart Infusion (BHI) agar supplemented with blood.

PCR analysis

Bacterial DNA from the 84 feces samples and 192 bacterial isolates was obtained using, respectively, the commercial QIAmp DNA Stool Mini Kit and QIAmp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions. DNA was stored at -80 °C until use.

The presence of *B. fragilis* in the fecal samples was detected by PCR amplification with 16S rRNA primers. DNA from the bacterial isolates was amplified with 16S-23S rRNA primers. All DNA samples positive for *B. fragilis* were screened for the presence of ETBF and/or NTBF sequences. The oligonucleotide pairs and amplification conditions used are described in Table 1.

All PCR assays were performed as follows: 1X PCR buffer, 50 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 mM each primer, 0.5 U of Platinum *Taq* polymerase (Invitrogen), and 1 ng of DNA. PCR products were analyzed by 1% agarose gel electrophoresis, stained with 0.5 μg/mL of ethidium bromide and photographed under UV light.

B. fragilis ATCC 43858 (ETBF pattern I, *bft*$^+$) and ATCC 25285 (NTBF pattern III, *bft*$^-$) were used, respectively, as positive and negative controls. The other strains used as controls were: *B. thetaiotaomicron* ATCC 29741, *B. vulgatus* ATCC 8482, *B. caccae* ATCC 43185, *B. ovatus* ATCC 8483, *B. eggerthii* ATCC 27754, *B. uniformis* ATCC 8492, *B. stercoris* ATCC 43183, *Parabacteroides distasonis* ATCC 8503, and *P. merdae* ATCC 43184.

Statistical analysis

The chi-square test was used to analyze the data on the presence of ETBF and NTBF strains, obtained by PCR and the culture method. The significance level was set at 5% using the BioEstat 2009 version 5.3.5.

Results

A total of 192 isolates were obtained from 84 children by culture method. Among those, 19 strains isolated from 8 (9.5%) children were identified as *B. fragilis* by 16S-23S rRNA PCR. None harbored the *bft* gene. Nine (47.3%) out of the 19 *B. fragilis* strains, isolated from 3 children, contained a 1.6-kb amplicon and therefore belonged to NTBF pattern III. The remaining 10 (52.7%) *B. fragilis* strains obtained from 5 children did not show DNA amplification and were considered belonging to NTBF pattern II (Table 2).

PCR analysis of the DNA isolated from the fecal samples showed that all 84 samples were positive for *B. fragilis* and four (4.7%) out of the 84 children were *bft*-positive. Twenty seven (32.1%) out of the 84 samples produced a 1.6-kb amplicon, thus belonging to NTBF pattern III, while 52 (61.9%) samples did not generate any DNA amplification products and thus were considered NTBF pattern II samples. In one fecal sample (1.2%), the presence of both ETBF and NTBF pattern III DNA sequences was observed. Moreover, statistical analysis did not show significant differences between the bacterial isolates and fecal samples in regards to the presence of NTBF pattern II ($p = 0.456$) and pattern III ($p = 0.209$) (Table 2).

Discussion

The impact of the resident microbiota on human health has been extensively discussed, and studies have suggested that alterations of the intestinal ecology might be associated with inflammatory bowel diseases, diabetes or obesity (Musso *et al.*, 2011; Shen *et al.*, 2012).

Microbial culture is considered the gold standard for detection of bacteria, but requires a high number of viable cells. Bacterial characterization by different methods, including DNA analysis, has also been used to elucidate microbiota-host interactions. In this study, 19 *B. fragilis* strains isolated from feces did not harbor the *bft* gene. Since ETBF strains are associated with acute diarrhea processes, their presence was not expected in children without diarrhea. Determination of the genetic patterns (I, II or III) was

Table 1 - Oligonucleotides and PCR conditions used to detect toxigenic and non-toxigenic *B. fragilis*.

Genes	Oligonucleotides	Amplification	Size	References
	5' → 3'	Conditions	(bp)	
		35 cycles		
B. fragilis	F: GTACACACCGCCCGT	94 °C x 30 s	420	(9)
	R: GCTAATCCCCCAATCATAC	62 °C x 30 s		
(16-23 rRNA)*		72 °C x 30 s		
B. fragilis	F: TCRGGAAGAAAGCTTGCT	35 cycles	162	(18)
(16S rRNA)**	R: CATCCTTTACCGGAATCCT	94 °C x 30 s		
		56 °C x 60 s		
		72 °C x 60 s		
bft gene	F: GACGGTATGTGATTTGTCTGAGAGA	35 cycles	294	(14)
	R: ATCCCTAAGATTTTATCCCAAGTA	94 °C x 30 s		
		52 °C x 60 s		
		72 °C x 60 s		
NTBF*** Patterns	F: TTCAACCTGATCGATCCGGAAGATCCG	29 cycles	1600 (pattern III) None (pattern II)	(5)
	R: GCTGGTAGACTACCTGAGTAAGGAGTC	94 °C x 60 s		
		66 °C x 2 min		
		72 °C x 60 s		

*Primer used to identify *B. fragilis* from bacterial isolates.
**Primer used to identify *B. fragilis* from fecal samples; Degenerate primer R = A/G.
***NTBF: Non-enterotoxigenic *Bacteroides fragilis*.

Table 2 - Presence of enterotoxigenic and non-enterotoxigenic *Bacteroides fragilis* in 19 bacterial isolates and 84 fecal samples.

B. fragilis	Bacterial isolates		Fecal samples		$p^{\S}$
	n	%	n	%	
ETBF*	0	0	4	4.7	-[a]
Pattern I					
NTBF**	10	52.6	52	61.9	0.456
Pattern II					
NTBF	9	47.4	27	32.1	0.209
Pattern III					
ETBF + NTBF III	0	0	1	1.2	-[a]

§Significance level for chi-square test. $p < 0.05$.
[a]Without sufficient sample to perform the chi-square test.
*ETBF: Enterotoxigenic *Bacteroides fragilis*.
**NTBF: Non-enterotoxigenic *Bacteroides fragilis*.

performed in this study by using DNA obtained from the bacterial isolates and feces. To achieve better accuracy and specificity in the NTBF pattern II determination, a 16S rRNA-specific primer pair to *B. fragilis* was used to evaluate all DNA samples.

Studies have shown the presence of ETBF in 2% to 3% of Brazilian children from 1 month to 12 years old without diarrhea (Bressane *et al.*, 2001; Kryzanowsky *et al.*, 2003). In our study, four (4.7%) out of the 84 samples harbored the *bft* gene and they originated from children older than 5 years of age, thus showing a slight increase of the ETBF rate, compared to the previous studies. Merino *et al.* (2011) showed no statistically significant difference in the incidence and number of ETBF strains detected in fecal samples from children with and without diarrhea.

B. fragilis strains harboring the *bft* gene have been associated with inflammatory bowel disease and colorectal cancer, and their phenotypic and genotypic features could be used to evaluate the involvement of these strains in various human disorders (Basset *et al.*, 2004; Prindiville *et al.*, 2000; Toprak *et al.*, 2006).

The *bft* gene is located in the 6-kb BfPAI flanked by a 12-kb region. Nucleotide analysis of the flanking region suggests that the BfPAI is a mobile genetic element and could be transferred from ETBF to NTBF strains. Since, the BfPAI or its flanking regions can be self-mobilized, and the presence of the 12-kb region observed in NTBF pattern III strains is suggestive of this event (Franco *et al.*, 1999), comprehensive evaluation of *B. fragilis* strains isolated from children without diarrhea is justified.

A high prevalence of NTBF pattern III strains has been observed in different geographic areas, such as Bangladesh and Korea (Franco *et al.*, 1999). In this study, 9 (47.4%) out of 19 NTBF strains belonged to pattern III and 10 (52.6%) belonged to pattern II. Our results also showed that 27 (32.1%) fecal samples contained NTBF pattern III DNA sequence, and 52 (61.9%) samples contained NTBF pattern II DNA sequence. One sample contained both ETBF and NTBF pattern III DNA sequence and should be further analyzed. There were differences in the prevalence of patterns II and III between the bacterial and fecal samples (Table 2). This could be explained by a lower number of samples positive for *B. fragilis* (8 samples) obtained by the culture-based technique. Although the NTBF pattern II was prevalent in both bacterial and fecal samples, our results showed a low rate of ETBF.

In a longitudinal study conducted during a period of one year, Zitomersky *et al.* (2011) observed the presence of ETBF in 6 out of 15 healthy individuals, of which 3 harbored only ETBF, while 2 harbored ETBF and NTBF during this period. The authors suggested that an individual can harbor more than one strain at the same time and toxigenic strains can persist in the intestinal ecosystem without causing acute damage to their host.

In conclusion, our results showed a low incidence of ETBF in the intestinal tract of children without diarrhea. However, a relatively high incidence of NTBF strains, especially those belonging to pattern III, suggests that further investigations are needed, specially due to the possibility that NTBF strains may become ETBF by acquiring the BfPAI. In addition, longitudinal studies must be conducted to investigate the persistence of ETBF strains in healthy subjects and their association with clinical conditions such as diarrhea, colitis and colorectal cancer.

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