PATHOGENESIS

# Prevalence of the *Bacteroides fragilis* Group and Enterotoxigenic *Bacteroides fragilis* in Immunodeficient Children

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Members of the *Bacteroides fragilis* group are indigenous to the human and animal intestinal microbiota and they are responsible for several endogenous infections. Enterotoxigenic *B. fragilis* (ETBF) has been associated with acute diarrhea in children and farm animals. Immunodeficient patients are more predisposed to different opportunistic infections, including anaerobic infections. In this study, 130 stool samples were analysed from 56 immunodeficient and 74 healthy children. Enterotoxin production was detected by cytotoxicity assay on HT-29 cells and by PCR. *B. fragilis sensu strictu* was prevalent in both groups and ETBF species was detected from a single stool sample belonged to an immunodeficient child with AIDS.

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## Introduction

*Bacteroides fragilis* is an important clinical pathogen and is the most common anaerobe isolated from human clinical specimens producing endogenous infections [1]. Myers *et al.* [2] reported that some strains of *B. fragilis* produce an enterotoxin associated with diarrhea in young farm animals, and that this could be detected in lamb ileal loop test. Enterotoxigenic *B. fragilis* (ETBF) has also been found to cause diarrhea in children and the role of these organisms as clinical pathogens in acute diarrhea or extra-intestinal infections, have also been mentioned in several studies [3,11].

Experimental methods have been employed to detect the enterotoxin from *B. fragilis* that induces a fluid response in ligated intestinal loop and a cytotoxic response on HT-29 cells, characterized by rounding cells and reorganization of F-actin [12]. The enterotoxin is a zinc-metalloprotease called fragilisyn with a molecular weight of 20,000 Da. It cleaves the extracellular matrix proteins, actin, gelatin, fibrinogen and collagen [13,15].

In recent years, PCR has been used for identification of several micro-organisms and it has also been used to detect enterotoxigenic *B. fragilis* [16].

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Immunodeficiency is defined as any alteration in the body defense, and is classified into two categories; primary and secondary. Primary immunodeficiency alterations occur due to the host's innate genetic factors whereas, secondary immunodeficiency can be produced by several factors such as age, infections, radiation and drug use [17]. Thus, these immunological alterations could contribute to the development of several infectious diseases, including anaerobic infections [18]. Coronado *et al.* [19], suggested that anaerobic infections may be closed as opportunistic and the treatment for these infections should be against these micro-organisms.

The goal of this study was the isolation and identification of bacteria of the *B. fragilis* group and the detection of the enterotoxigenic *B. fragilis* from feces of immunodeficient children.

## Materials and Methods

### Patients

Fifty-six hospitalized immunodeficient children, aged 1 month to 12 years old, without sex or race distinction were selected: 32 children with AIDS and 24 with other immunodeficiencies (ten were receiving cancer treatment with chemotherapy and radiotherapy, three had malnutrition, eight were receiving corticoids treatment and three had primary immunodeficiency). The hospitalized children were divided in two groups according to the presence or absence of diarrhea, for sample collection. Also, 74 healthy children from two different day care centres, with no immune alteration and in the same age group, were selected as control. The Ethic Commission from both hospitals approved this project.

### Bacterial isolates

Fresh stool samples from immunodeficient patients with or without diarrhea and from control group were naturally obtained and immediately plated onto a selective *Bacteroides fragilis* — bile — esculin agar (BBE) [20] and incubated in 90% $N_2$/10% $CO_2$, at 37°C, for 72 h. Characteristic dark colonies were subcultured on blood agar and then identified by conventional biochemical tests or by using kits for identification API 32-A (bioMérieux). All the isolates belonged to the *B. fragilis* group were stored in skim milk at −80°C.

Two enterotoxin-positive strains were used as control: *B. fragilis* ATCC 43858 and *B. fragilis* GAI 97124, generously provided by Dr Annalisa Pantosti, from Laboratory of Bacteriology and Medical Mycology, Institute Superiore di Sanitá, Rome, Italy and by Dr Naoki Kato from Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan, respectively.

### Enterotoxin production

Bacteria of the *B. fragilis* group were grown in brain heart infusion broth (BHI, Difco Laboratories) supplemented with 0.5% yeast extract incubated in anaerobiosis, at 37°C, for 48 h. The culture was centrifuged ($13,000 \times g$, 5 min) and the pellet and supernatant were separately collected and stored at −20°C [3].

### Cytotoxicity assay

HT-29 cells were grown in flask with 50 mL of Eagle medium (Gibco BRL, Life Technologies) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin and heat-inactivated fetal bovine serum (15%), incubated in air with 5% $CO_2$, at 37°C. Cells were transferred to a fresh medium with 1 : 2 dilutions and resuspended in 20 mL of medium. Then, cells were distributed (200 μL/well) into a 96-well microtitration plate (Corning, U.S.A.) and allowed to growth for 2–3 days until discrete cell clusters were visible. Before the assay, the medium was removed and 180 μL of fresh medium without serum was added and 20 μL of supernatant were inoculated into each well, in duplicate. The plate was incubated with 5% $CO_2$, at 37°C, and examined after 3–4 h for the presence of the typical toxin-induced cellular cytotoxic changes [3].

### DNA extraction

Pellets were allowed to thaw and were then mixed with 500 μL of Milli-Q water and washed twice at $12,000 \times g$, for 10 min. Pellet was resuspended in 500 μL of Milli-Q water and boiled for 10 min. After centrifugation ($14,000 \times g$, 10 min) the supernatant was saved and transferred to a new tube and used as template.

### PCR amplification

The primers used in this study were synthesized according to Pantosti *et al.* [16]. Forward primer BF1 (5′-GAG GGT GTA TGT GAT TTG TCT GAG AGA-3′) and reverse primer BF2 (5′-ATC CCT AAG ATT TTA TTA TCC CAA GTA-3′) were produced in the Biotechnology Branch, Centers for Disease Control and Prevention (CDC, Atlanta, GA, U.S.A.). This specific primer pair amplified a characteristic 294-bp band and the PCR assay was performed as described earlier [16]. DNA amplification was performed in

25 µL containing 2.5 µL of 10 X PCR buffer (Gibco), 1.25 µL of MgCl$_2$ (1.5 mM), 2.0 µL of dNTP mixture (0.2 mM) (Gibco), 0.25 µL of *Taq* DNA polymerase (0.5 U) (Gibco), 1 µL of each primer (0.4 µM), 7 µL of ultrapure water (Milli-Q plus) and 10 µL of DNA template. Amplification was performed in a DNA thermal cycler (Perkin Elmer, Amp PCR System 2400) programmed for 94°C (5 min) followed by 35 cycles of 94°C (1 min), 52°C (1 min), 72°C (1 min), and then 72°C (5 min) to allow the completion of DNA extension. A negative control without template DNA was included in each PCR run.

### Detection of amplified products

Amplified products were visualized by electrophoresis in 1.6% agarose gel in 1X TBE buffer (1M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4), at 80 V, for 2 h, and then stained with ethidium bromide (0.5 mg/mL). DNA bands were photographed on a UV light transilluminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science). Molecular mass standard 50 bp DNA Ladder (Gibco) was included.

### Statistical analyses

$\chi^2$ tests were used to check differences in isolation among bacteria of the *B. fragilis* group from patients and control.

## Results

*Recovery of bacteria of the B. fragilis group and ETBF from feces of children*

A total of 155 strains of the *B. fragilis* group were isolated: 76 strains from 56 immunodeficient patients and 83 strains from 74 children of control group.

Table 1 shows the incidence of bacteria of the *B. fragilis* group and *B. fragilis sensu strictu* in hospitalized patients with or without diarrhea, and in control group. ETBF bacteria (3.1%) were isolated from one single stool sample belong to an immunodeficient patient with AIDS. Species of the *B. fragilis* group were observed in 32 children with AIDS, seven (21.9%) with diarrhea and six (18.8%) without diarrhea. *B. fragilis sensu strictu* were isolated in four (12.5%) children with diarrhea and in four (12.5%) without diarrhea.

Also, from 24 children selected with other immunodeficiencies, five (20.8%) without diarrhea showed species of the *B. fragilis* group, and *B. fragilis sensu strictu* was isolated from two (8.3%) patients. In addition, from children of the control group, 37 (50%) species of the *B. fragilis* group were isolated, including 20 (27%) samples positive to *B. fragilis sensu strictu* (Table 1).

On the other hand, from immunodeficient children with AIDS, 29 (45.3%) *B. fragilis*, seven (11%) *B. distasonis*, eight (12.5%) *B. vulgatus*, 13 (20.3%) *B. ovatus*, three (5%) *B. thetaiotaomicron*, two (3.1%) *B. eggerthii*, two (3.1%) *B. uniformis* were identified. Patients with different problems than AIDS harbored four (33.3%) *B. fragilis*, three (25.0%) *B. distasonis* and *B. vulgatus* and two (16.7%) *B. thetaiotaomicron* (Table 2). Also, from control group the following species were isolated: 49.4% *B. fragilis*, 20.2% *B. distasonis*, 15.7% *B. vulgatus*, 4.8% *B. ovatus*, 3.6% *B. thetaiotaomicron*, 2.4% *B. eggerthii*, and 1.2% *B. uniformis*, *B. caccae* and *B. stercoris*.

### Enterotoxin detection

All the 155 isolated strains recovered from patients and healthy children were examined for enterotoxin production by cytotoxicity assay with HT-29 cell and PCR.

ETBF were isolated and detected from only a single stool sample. From this analysed sample, eight strains

**Table 1.** Incidence of bacteria of the *Bacteroides fragilis* group, *B. fragilis sensu strictu*, and enterotoxigenic *B. fragilis* (ETBF) from 56 immunocompromised and 74 control children

| | AIDS (*n* = 32) | | | | | | Another*(*n* = 24) | | | | | | Control (*n* = 74) | |
| | Diarrhea | | No diarrhea | | Total | | Diarrhea | | No diarrhea | | Total | | | |
| Bacteria | No. | (%) | No. | (%) | No. | (%) | No. | (%) | No. | (%) | No. | (%) | No. | (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. fragilis* group | 7 | (21.9) | 6 | (18.8) | 13 | (40.6)[a] | 0 | (0) | 5 | (20.8) | 5 | (20.8)[b] | 37 | (50)[c] |
| *B. fragilis sensu strictu* | 4 | (12.5) | 4 | (12.5) | 8 | (25)** | 0 | (0) | 2 | (8.3)** | 2 | (8.3) | 20 | (27)** |
| ETBF | 0 | (0) | 1 | (3.1) | 1 | (3.1)** | 0 | (0) | 0 | (0) | 0 | (0) | 0 | (0) |

*Other immunodeficiencies: cancer in treatment with chemotherapy and radiotherapy, malnutrition, treatment with corticoids and primary immunodeficiency.

**No significant difference.

a × b = $P < 0.05$.

a × c = $P > 0.05$.

b × c = $P < 0.05$.

**Table 2.** Incidence of bacteria of the *Bacteriodes fragilis* group isolated from stool samples from patients and control group

| Species | AIDS | | Other* | | Total of patients | | Control | |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % |
| *B. fragilis* | 29 | 45.3 | 4 | 33.3 | 33 | 43.4 | 41 | 49.4 |
| *B. distasonis* | 7 | 11 | 3 | 25 | 10 | 13.2 | 17 | 20.2 |
| *B. vulgatus* | 8 | 12.5 | 3 | 25 | 11 | 14.5 | 13 | 15.7 |
| *B. ovatus* | 13 | 20.3 | 0 | 0 | 13 | 17.1 | 4 | 4.8 |
| *B. thetaiotaomicron* | 3 | 4.7 | 2 | 16.7 | 5 | 6.6 | 3 | 3.6 |
| *B. eggerthii* | 2 | 3.1 | 0 | 0 | 2 | 2.6 | 2 | 2.4 |
| *B. uniformis* | 2 | 3.1 | 0 | 0 | 2 | 2.6 | 1 | 1.2 |
| *B. caccae* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1.2 |
| *B. stercoris* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1.2 |
| Total | 64 | 100 | 12 | 100 | 76 | 100 | 83 | 100 |

*Other immunodeficiencies: cancer in treatment with chemotherapy and radiotherapy, malnutrition, treatment with corticoids and primary immunodeficiency.

were isolated: six *B. fragilis* (enterotoxin-positive) and two *B. vulgatus* (enterotoxin-negative). They were detected by cytotoxicity assay with HT-29 and by PCR. Children from control group did not harbor ETBF species.

## Discussion

*B. fragilis sensu strictu* is emerging as an etiologic agent of diarrhea in farm animals and in humans [2,4,10]. Recently, Pantosti *et al.* (16) detected ETBF by PCR from a little portion of the positive stool specimens for these organisms by culture. Detection of ETBF species in stools depends on the amount of toxin produced, sensibility of the assay, and stability of the toxin, which is susceptible to degradation by proteases [12,15].

Currently, identification of enterotoxin production is achieved by culturing in selective medium (BBE) and testing the isolates for the presence of enterotoxin by the cytotoxicity assay with HT-29 cells [21] or lambs ileal loop test [4] and more recently by PCR [16]. The HT-29 cytotoxicity assay is used for detecting the phenotypic characteristic, but it is time-consuming [21]. On the other hand, PCR assay detects the genotypic characteristics and it is a sensible method of detection. However, both of methods can be used as control for ETBF detection [16].

In this study we evaluated the isolation and identification of bacteria of the *B. fragilis* group and the presence of ETBF in stool samples from hospitalized immunodeficient children. It is known that indigenous microbiota is an important factor for regulation or colonization of opportunistic pathogens but, information about their regulatory and/or deleterious effects to the host is still not clear [22]. Immunocompromised patients are more susceptible to several infections, including enteric infections by several organisms [23]. Coronado *et al.* [19] showed

that patients with AIDS have a high risk of developing anaerobic infections.

A significant difference was observed ($P < 0.05$) between isolation of species of the *B. fragilis* group among patients with immunodeficiencies other than AIDS and the control group. Also, a significant difference amongst the other immunodeficiencies group and the AIDS group was observed. On the other hand, there were not differences in the isolation of the *B. fragilis sensu strictu* from all children groups ($P > 0.05$). Also, no significant difference in the ETBF isolation and control group was observed ($P > 0.05$) (Table 1).

All the studied patients were on antimicobial agents at the time of sampling, and it could be that this altered the bacterial isolation. On the other hand, it is suggested that some factors such as immunological alterations, age, nutritional conditions, genetic factors, pathologies or antimicrobial therapy, can also interfere on the *B. fragilis* isolation [5,10].

Bacteria of the *B. fragilis* group isolated from the control group were isolated in 50% samples and *B. fragilis sensu strictu* was isolated in 27%. These results are in accordance with San Joaquin *et al.* [7], who showed the isolation of this species in 21% stool samples from control group. Similar data has also been observed in Italian children (46%) and in Apache American children (50%) [5,6].

In both immunodeficient and control groups, the *B. fragilis sensu strictu* was the most predominant. On the other hand, studies performed with adults showed the predominance of *B. distasonis*, *B. vulgatus* and *B. fragilis*. It suggests that some factors such as age and/or immunological factors can produce any alteration in host's indigenous microbiota [24].

ETBF were isolated and detected only from a single stool sample belonging to an AIDS child, without diarrhea at the moment of sampling, but he had diarrhea three days prior to collection. In addition, no other enteropathogenic organism was observed in

stool cultures but *Endolimax nana* and *Blastocystis hominis* were observed. On the other hand, ETBF from this patient could represent a small fraction of his intestinal microbiota or could be the agent of the diarrhea prior to sample collection or may be transmitted by a fecal–oral contamination, as suggested by Shoop *et al.* (1990).

The presence of ETBF have been associated with acute diarrhea in children older than one year old in USA, Italy, Sweden and Japan [5–7,10] and recently, in Nicaraguan children younger than one year old [9]. Also, these studies showed that patients with or without diarrhea could harbor ETBF organisms in their intestinal tracts, suggesting that ETBF might be present in small numbers in human intestinal indigenous microbiota [5].

Our results also suggest that the frequency of bacteria of the *B. fragilis* group, *B. fragilis sensu strictu* and ETBF strains from stool samples can be influenced for the selected population or by several factors such as diet, socio-economic conditions, host's susceptibility, microbial genetic or clinical sample collection [10,18]. The presence of ETBF as a possible agent of acute diarrhea is suggested in several countries. However, our results show that the presence of ETBF was not significant in immunodeficient patients, and it suggests that other enteropathogens such as rotavirus, EPEC, ETEC or *Vibrio chloreae* could be implicated in diarrheal processes in Brazil. Also, these data suggest further studies about prevalence of ETBF in immunocompromised patients in different countries, particularly where a high incidence of this bacterial group is observed. In addition, these results indicate the need of more studies to evaluate the role of each bacteria of the *B. fragilis* group (*B. fragilis sensu strictu* and ETBF) play in the diarrheal processes in different hosts. Certainly, it could provide a better understanding of these infections in ecological and pathogenic terms.

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