Rev. Inst. Med. trop. S. Paulo
45(4):225-227, July-August, 2003

BRIEF COMMUNICATION

DETECTION OF NON-ENTEROTOXIGENIC AND ENTEROTOXIGENIC *Bacteroides fragilis* IN STOOL SAMPLES FROM CHILDREN IN SÃO PAULO, BRAZIL

Flávio KRZYZANOWSKY & Mario J. AVILA-CAMPOS

SUMMARY

Non-enterotoxigenic bacteria of the *Bacteroides fragilis* group and enterotoxigenic *B. fragilis* were identified from children with and without aqueous acute diarrhea. In this study, 170 stool samples from 96 children with and 74 without diarrhea were analyzed. Enterotoxin production and the toxin gene detection were detected by cytotoxicity assay on HT-29/C_1 cells and by PCR, respectively. *B. fragilis* species was prevalent in both groups and enterotoxigenic *B. fragilis* strains were isolated from two children with diarrhea. More studies are important to evaluate the role of each bacteria of the *B. fragilis* group, including enterotoxigenic strains play in the diarrheal processes in children.

KEYWORDS: *Bacteroides fragilis* group; ETBF; Acute diarrhea; Children.

Bacteroides fragilis is considered to be an important member of the human intestinal indigenous microbiota and in various experimental animal models *B. fragilis* has been reported to be more virulent than the other species from the *B. fragilis* group[3].

The involvement of *B. fragilis* in the etiology of human and animal diarrhea has been the subject of some investigations in the last years[6,11]. Childhood diarrhea is one of the most important causes of morbidity and mortality worldwide[5]. MYERS *et al*.[10] reported *B. fragilis* strains producing an enterotoxin (ETBF) that causes diarrhea in young farm animals and which is associated with diarrhea in man[2,7,14,15].

The enterotoxin or fragilisyn has been characterized as a zinc-metalloprotease with a molecular weight of 20,000 Da, and with activity against tight junction proteins, disrupting the intestinal epithelial barrier[9,12,13] and inducing fluid losses[19]. In this study, the isolation and identification of bacteria of the *B. fragilis* group and the detection of the ETBF in stool samples of children with and without diarrhea were performed.

Fresh stool samples from ninety-six hospitalized children with aqueous acute diarrhea from two hospitals (Menino Jesus Hospital and Instituto da Criança, São Paulo, SP, Brazil) and from 74 healthy children from two different day care centers, aged one month to 12 years old, without sex distinction were analyzed. The Ethic Commission from both hospitals approved this study. Collected stools from children with or without diarrhea were immediately plated onto a selective *B. fragilis*-bile-esculin agar[8] (BBE, Difco Laboratories) and incubated in atmosphere with 90% N_2/10% CO_2 at 37 °C, for 72 hours. Characteristic dark colonies grown in BBE were subcultured on blood agar (BHI agar - Difco, enriched with 5% sheep desfibrined blood). Isolates were identified by using identification kits API 32-A (bioMérieux) and stored in 10% skim milk at -70 °C.

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

Bacteria from *B. fragilis* group were grown in brain heart infusion broth (BHI, Difco) supplemented with 0.5% yeast extract (Difco) in anaerobiosis, at 37 °C, for 48 hours and centrifuged (13,000 *g,* 5 minutes). Supernatant and pellet were separately collected and stored at -20 °C. Cytotoxic assay was performed according BRESSANE *et al*.[1] in HT-29/C_1 cells grown in Eagle medium (Gibco BRL, Life Technologies) supplemented with 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1} and heat-inactivated fetal bovine serum (15%) in air plus 5%-CO_2, at 37 °C. Then, 20 µl of bacterial supernatant were inoculated into each well, in duplicate, and plates were incubated (air- 5%-CO_2, at 37 °C), and examined after 3-4 hours for the presence of the typical toxin-induced

Department of Microbiology, Institute of Biomedical Science, University of São Paulo, São Paulo, SP, Brazil.
Correspondence to: Dr. Mario J. Avila-Campos, Anaerobe Laboratory, Department of Microbiology, ICB, University of São Paulo, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brazil. Phone: +55-11-3091-7344; Fax: + 55-11-3091-7354. E-mail: mariojac@usp.br

cellular alterations[14]. Bacterial pellets mixed with 500 µl of Milli-Q water, washed twice (12,000 *g*, for 15 minutes), and resuspended in 500 µl of Milli-Q water were used for DNA extractions by boiling for 10 minutes. After centrifugation (14,000 *g*, 10 minutes) the supernatant was saved and used as template. Primers were synthesized according to PANTOSTI *et al*.[16] at the Biotechnology Branch, Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). For positive isolates the specific primer pair amplified a characteristic 294-bp fragment. DNA amplifications were 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 mM), 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 (five minutes) followed by 35 cycles of 94 °C (one minute), 52 °C (one minute), 72 °C (one minute), and then 72 °C (five minutes). A negative control without template was included in each PCR run. Amplified products were visualized by electrophoresis in 1.6% agarose gel in 1X TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4), at 80 v, for two hours. A 50 bp DNA Ladder (Gibco) was used as a molecular mass marker. Gels were stained with ethidium bromide (0.5 µg ml^{-1}) and photographed on a UV light transilluminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science). Bacterial isolation from both patient and control groups was analyzed by using a χ^2 test.

The incidence of bacteria of the *B. fragilis* group, non-enterotoxigenic *B. fragilis* (non-ETBF), and ETBF isolated from hospitalized children with aqueous acute diarrhea and from healthy children without diarrhea can be observed in Table 1. 0nly children with diarrhea harbored ETBF species (2.08%). Non-ETBF *B. fragilis* were isolated in 11 (11.45%) children with diarrhea and in 18 (24.3%) without diarrhea (Table 1). Also, the bacterial species that belonged to *B. fragilis* group isolated from children with and without diarrhea are shown in Table 2. All the recovered bacteria from children with and without diarrhea were examined for enterotoxigenicity on HT-29/C_1 cell and by PCR, and only ETBF were toxigenic and produced a characteristic 294 bp fragment. ETBF were isolated and detected in two children with diarrhea (8 months and 4 years old). No healthy children without diarrhea harbored ETBF.

B. fragilis species are emerging as etiologic agents of diarrhea in farm animals and humans[11]. ETBF detection from stools is amount-dependent of produced toxin, assay sensitivity, and toxin stability, but the toxin is susceptible for protease action[13].

Currently, the identification of enterotoxin production is achieved by culturing in selective medium (BBE) and by testing the isolates for the presence of enterotoxin by the cytotoxic assay with HT-29/C1 cells[15] or lambs ileal loop test[11]. In this study, the bacterial isolation from both patient and control groups was not significant ($P > 0.05$, Table 1). Hospitalized children were not using antimicrobial agents at the time of sampling. Although, it is suggested that some factors such as immunological alterations, age, nutritional conditions, genetic factors or pathologies could interfere in the *B. fragilis* isolation[4,20].

Bacteria of the *B. fragilis* group were isolated from 50% control group, in accordance with similar studies in Italian children (46%) and in Apache American children (50%)[15,17]. Non-ETBF was observed in 24.3% of this control group. The presence of ETBF has been associated with acute diarrhea in children older than one year-old in USA, Italy, Sweden and Japan[15,17,18,20], and recently in Nicaraguan children younger than one-year old[2]. Also, these studies showed that patients with or without diarrhea could harbor ETBF in their intestinal tract, but these organisms might be in a small number in the human intestinal indigenous microbiota[15]. In our study, ETBF were isolated and detected from two children with diarrhea and its presence was not significant, however, other enteropathogens such as rotavirus, EPEC, ETEC, or *Vibrio cholerae* were not found, but they could be implicated in diarrhoeal processes in Brazil[1]. Moreover, these results indicate the need of more studies to evaluate the role that each bacteria of the *B. fragilis* group, including non-ETBF and ETBF, play in the childhood diarrhea.

Table 1
Incidence of the *B. fragilis* group, non-ETBF and ETBF in stool samples obtained from 96 patients and 74 healthy children samples

Isolates	With diarrhea (96)		Without diarrhea (74)	
	No.	%	No.	%
B. fragilis group	16	16.6[a]	37	50[b]
Non-ETBF	11	11.45*	18	24.3*
ETBF	2	2.08*	0	0

*No significant difference; a x b = $P < 0.05$.

Table 2
Occurrence of bacteria of the *B. fragilis* group in 170 stool samples from children with and without aqueous acute diarrhea

Bacterial Isolates	Diarrhea		No diarrhea	
	No.	%	No.	%
Non-ETBF	67	72.82	42	53.84
ETBF	2	2.17	0	0
B. distasonis	11	11.95	17	21.79
B. uniformis	7	7.6	1	1.28
B. vulgatus	4	4.34	13	16.66
B. thetaiotaomicron	1	1.08	1	1.28
B. ovatus	0	0	4	5.12
Total	92	100	78	100

RESUMO

Detecção de *Bacteroides fragilis* enterotoxigênicos e não enterotoxigênicos de amostras fecais de crianças em São Paulo, Brasil

Bactérias não enterotoxigênicas do grupo *Bacteroides fragilis* e *B. fragilis* enterotoxigênicas foram isoladas e identificadas de crianças com e sem diarréia aguda aquosa. Neste estudo, 170 amostras fecais de 96 e 74 crianças, com e sem diarréia, respectivamente, foram analisadas. A produção de enterotoxina e a detecção do gene que media a produção da toxina foram determinadas por ensaios citotóxicos em células HT-29/C_1 e por PCR, respectivamente. A espécie *B. fragilis* foi prevalente em ambos

os grupos, e cepas de *B. fragilis* enterotoxigênicas foram isoladas de duas crianças com diarréia aquosa aguda. Maiores estudos são necessários para avaliar o papel de cada bactéria desse importante grupo bacteriano, incluindo-se o papel que as cepas enterotoxigênicas, desempenham no processo diarréico em crianças.

ACKNOWLEDGEMENTS

We thank Dr Leonard W. Mayer for providing the primers and for his critical review of this paper, and Marcela Alves Bressane and Zulmira Alves de Souza for their technical support. Flávio Krzyzanowski was supported by a Grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Proc. N. 135623/99-5. This study was partially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Proc. No. 99/06475-1.

REFERENCES

1. BRESSANE, M.A.; DURIGON, L.E. & AVILA-CAMPOS, M.J. - Prevalence of the *Bacteroides fragilis* group and enterotoxigenic *Bacteroides fragilis* in immunodeficient children. **Anaerobe, 7:** 277-281, 2001.

2. CÁCERES, M.; ZHANG, G.; WEINTRAUB, A. & NORD, C.E. - Prevalence and antimicrobial susceptibility of enterotoxigenic *Bacteroides fragilis* in children with diarrhoea in Nicaragua. **Anaerobe, 6:** 143-148, 2000.

3. DUERDEN, B.I. - Virulence factors in anaerobes. **Clin. infect. Dis., 18**(suppl.)**:** S253-S259, 1994.

4. FINEGOLD, S.M. - Host factors predisposing to anaerobic infections. **FEMS Immunol. med. Microbiol., 6:** 159-163, 1993.

5. HINES, J. & NACHAMKIN, I. - Effective use of the clinical microbiology for diagnosing diarrheal diseases. **Clin. infect. Dis., 23:** 1292-1301,1996.

6. KATO, N.; KATO, H.; WATANABE, K. & UENO, K. - Association of enterotoxigenic *Bacteroides fragilis* with bacteremia. **Clin. infect. Dis., 23**(suppl.)**:** S83-S86, 1996.

7. KATO, N.; LIU, C.; KATO, H. *et al.* - Prevalence of enterotoxigenic *Bacteroides fragilis* in children with diarrhea in Japan. **J. clin. Microbiol., 37:** 801-803, 1999.

8. LIVINGSTON, S.J.; KOMINOS, S.D. & YEE, R.B. - New medium for selection and presumptive identification of *Bacteroides fragilis* group. **J. clin. Microbiol., 7:** 448-453, 1978.

9. MONCRIEF, J.S.; OBISO Jr., R.J.; BARROSO, L.A. *et al.* - The enterotoxin of *Bacteroides fragilis* is a metalloprotease. **Infect. Immun., 63:** 175-181, 1995.

10. MYERS, L.L.; FIREHAMMER, B.D.; SHOOP, D.S. & BORDER, M.M. - *Bacteroides fragilis:* a possible cause of acute diarrheal disease in newborn lambs. **Infect. Immun., 44:** 241-244, 1984.

11. MYERS, L.L.; SHOOP, D.S.; STACKHOUSE, L.L. *et al.* - Isolation of enterotoxigenic *Bacteroides fragilis* from human with diarrhea. **J. clin. Microbiol., 25:** 2330-2333, 1987.

12. OBISO Jr., R.J.; LYERLY, D.M.; VAN TASSELL, R.L. & WILKINS, T.D. - Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage *in vivo***. Infect. Immun., 63:** 3820-3826, 1995.

13. OBISO Jr., R.J.; BEVAN, D.R. & WILKINS, T.D. - Molecular modeling and analysis of fragilysin, the *Bacteroides fragilis* toxin**. Clin. infect. Dis., 25** (suppl. 2)**:** S153-S154, 1997.

14. PANTOSTI, A.; CERQUETTI, M.; COLANGELI, R. & D'AMBROSIO, F. - Detection of intestinal and extra-intestinal strains of enterotoxigenic *Bacteroides fragilis* by the HT-29 cytotoxicity assay. **J. med. Microbiol., 41**: 191-196, 1994.

15. PANTOSTI, A.; MENOZZI, M.G.; FRATE, A. *et al*. - Detection of enterotoxigenic *Bacteroides fragilis* and its toxin in stool samples from adults and children in Italy**. Clin. infect. Dis., 24:** 12-16, 1997a.

16. PANTOSTI, A.; MALPELI, M.; WILKIS, M.; MENOZZI, M.G. & D'AMBROSIO, F. - Detection of enterotoxigenic *Bacteroides fragilis* by PCR. **J. clin. Microbiol., 35:** 2482-2486, 1997b.

17. SACK, R.B.; MYERS, L.L.; HILL, J. *et al.* - Enterotoxigenic *Bacteroides fragilis:* epidemiologic studies of its role as a human diarrhoeal pathogen. **J. Diarrhoeal Dis. Res., 10:** 4-9, 1992.

18. SAN JOAQUIN, V.H.; GRIFFIS, J.C.; LEE, C. & SEARS, C.L. - Association of *Bacteroides fragilis* with childhood diarrhea. **Scand. J. infect. Dis., 27:** 211-215, 1995.

19. VAN TASSEL, R.L.; LYERLY, D.M. & WILKINS, T.D. - Purification and characterization of an enterotoxin from *Bacteroides fragilis.* **Infect. Immun., 60:** 1343-1350, 1992.

20. ZHANG, G.; SVENUNGSSON, B.; KÄRNELL, A. & WEINTRAUB, A. - Prevalence of enterotoxigenic *Bacteroides fragilis* in adult with diarrhea and healthy controls. **Clin. infect. Dis., 29:** 590-594, 1999.

Received: 25 April 2003
Accepted: 05 June 2003