

Role of Enterotoxigenic *Bacteroides fragilis* in Children Less Than 5 Years of Age With Diarrhea in Tabriz, Iran

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Abstract

Background: Diarrhea is the most frequent health problem among children in developing countries. Defining the etiology of acute diarrhea is critical to disease therapy and prevention. Some anaerobic bacteria such as Enterotoxigenic *Bacteroides fragilis* (ETBF) strains cause diarrheal disease by production of enterotoxin in children less than 5 years old.

Objectives: This study aimed to evaluate the prevalence of ETBF among common bacteria and viruses causing diarrhea in children aged less than five years.

Materials and Methods: One hundred diarrheal stools were cultured for detection of aerobic and anaerobic pathogen bacteria by direct plating on selective media and antibiotic susceptibility tests were performed according to clinical and laboratory standards institute (CLSI) guidelines on isolates of ETBF. The enterotoxigenic gene among *B. fragilis* isolates was also investigated using the polymerase chain reaction (PCR) method. Detection of viral pathogens was carried out using the latex agglutination test.

Results: Ten *B. fragilis* were isolated from 100 diarrheal fecal specimens. All isolates were susceptible to metronidazole, while 10% were susceptible to clindamycin. Four (40%) ETBF were isolated. Rotaviruses (57.2%) and adenoviruses (18.6%) were the most frequently detected etiological agents.

Conclusions: ETBF is one of the etiological agents that may cause diarrhea in children but it is not the commonest of them. Metronidazole is still an effective antibiotic against *B. fragilis*. Viruses are the most important etiological agents of diarrhea in children less than 5 years of age.

Keywords: Diarrhea, Susceptibility Pattern, PCR, Rotavirus, Adenovirus, *Bacteroides fragilis*

1. Background

Infective diarrheal disease is a major public health problem throughout the world and a leading cause of morbidity and mortality among children. It can be caused by a wide range of bacteria, viruses, and parasites (1). Every child under 5 years of age experiences about three incidents of diarrhea per year in the developing world (2). More than 200 viral, bacterial, and parasitic pathogens of diarrhea have been recognized to date, but only a few etiological agents cause the enormous majority of diarrheal diseases in children (2). These include rotavirus, adenoviruses, *Escherichia coli*, *Campylobacter jejuni*, *Shigella* spp., non-typhoidal *Salmonella*, *Giardia lamblia*, *Cryptosporidium* spp., enterotoxigenic *Bacteroides fragilis* and *Entamoeba histolytica* (2-5). Unluckily, a large number of cases of diarrheal disease are of unidentified etiology. There are several

reasons for this, including weakness of causative pathogenesis, demanding growth requirements, and lack of acknowledgment of some organisms as enteric pathogens (2, 6).

Some of the anaerobic bacteria, such as *B. fragilis*, can also cause diarrhea in children. *Bacteroides fragilis* is an anaerobic Gram-negative bacillus and a member of the normal flora community of the human gastrointestinal tract and vagina. *B. fragilis* represents a common cause of endogenous infections in humans that is frequently associated with polymicrobial infections such as intra-abdominal, diabetic foot, obstetric-gynecologic tract and surgical site infections, as well as toxin-dependent diarrhea in adults and children (7, 8). Enterotoxigenic *Bacteroides fragilis* (ETBF) induced diarrhea in children has been reported by different researchers in various parts of the world, but ETBF has also been found in non-diarrheal

fecal samples (9, 10).

Enterotoxigenic *Bacteroides fragilis* (ETBF) strains produce a 20 kDa zinc metalloprotease called fragilisyn. ETBF has been described as causing diarrheal disease in animals and particularly in human children. This toxin results in swelling and rounding of the cultured enteric cell line (11, 12).

There is evidence to confirm that antimicrobial agents can decrease the severity and duration of some intestinal infections, chiefly in those bacterial infections that cause acute watery diarrhea. There are several antibiotics that have been studied in the management of infectious diarrhea, some empirical and some targeted (13). Due to the length of time required to culture, isolate and determine the sensitivity of anaerobic isolates to different antibiotics and the high costs of these procedures, anaerobic infections are often treated empirically, based on surveillance reports of susceptibility patterns of these pathogens. Susceptibility varies considerably among the different species in the group (14).

In recent years, there has been an increasing resistance among *B. fragilis* to a wide range of antibacterial agents including metronidazole (MTZ), clindamycin (CD), β -lactams and others agents (15-17). The increasing resistance to different antibiotics in clinical isolates of *B. fragilis* leads to limitation of therapeutic alternatives in the treatment of infection caused by this organism. Susceptibility to antibiotic agents varies among anaerobic bacteria according to geographical region, and in most cases, even from one hospital to other hospitals in the same region (14).

2. Objectives

This study aimed to assess the role of ETBF among the most common pathogens causing diarrhea in children and to determine minimum inhibitory concentration (MIC) for metronidazole and clindamycin in these isolates.

3. Materials and Methods

3.1. Sampling

In a descriptive study, 100 diarrheal fecal samples were collected from outpatient and hospitalized children that were sent consecutively to the laboratory of pediatric education and medical center by physicians. Specimens were examined directly for vegetative forms of parasites and cysts by standard microscopy with iodine-stained wet-mount preparations (18). Stool samples were cultured on Hecktoen Enteric agar (Merck co, Germany) for *Shigella*

and *Salmonella* strains and MacConkey agar (Merck co, Germany) for detection of *E. coli*. Bacterial isolates were identified according to the standard microbiological procedures (3). For isolation of *B. fragilis*, specimens were cultured in Bacteroides Bile Esculine agar (BBE, Himedia Laboratories Pvt. Ltd., India) and Kanamycin-Vancomycin-Laked Blood (KVLB, Basal Medium is Brucella agar (Fluka Chemie AG CH-9471 Buchs, Switzerland) media, and incubated at 37°C for 48 hours in an anaerobic atmosphere ($H_2 = 10\%$, $N_2 = 80\%$, $CO_2 = 10\%$) using Mart jars (MART Microbiology, B.V. The Netherlands) and the Anoxomat system (19).

Suspicious 1 mm colonies with black surroundings (showing esculin hydrolysis) were selected in BBE medium and further identified after conducting an anaerobic tolerance test using MID8 (Mast Identification 8, according to the manufacturer's instructions) and also some biochemical tests such as catalase and indole production and sugar fermentation (sucrose, arabinose, xylose, and rhamnose) (19).

3.2. Rotavirus and Adenovirus Latex Agglutination Test

Commercial latex agglutination test (Biomérieux, France) for the detection of *Rotavirus* and *Adenovirus* in human feces was used according to the manufacturer's instructions. In this test latex particles, coated with monoclonal antibodies directed towards specific *Rotavirus* and *Adenovirus* antigens, cross-link in the presence of *Rotavirus* and *Adenovirus* antigens, resulting in clearly visible agglutination. In the absence of these viral antigens, the particles remain in smooth suspensions (20).

3.3. Determination of Antibiotic Sensitivity

Etest strips (Liofilchem co. Italy) were used on brucella agar supplemented with 5% sheep blood for MIC determination of metronidazole and clindamycin against *B. fragilis* isolates. Plates were incubated anaerobically at 37°C for 48 hours and results were interpreted by clinical and laboratory standards institute (CLSI) guidelines (21).

3.4. DNA Extraction

One loopful of cultured *B. fragilis* was suspended in 300 μ L of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and placed at 80°C for 20 minutes to kill the bacteria. DNA was extracted by CTAB, SDS and proteinase K after sedimentation with isopropanol and washing with ethanol 70% extracted DNA was resolved in 100 μ L TE buffer (22).

3.5. PCR Method

Specific primers of 294 bp fragment (BF1:5'dGACGGTGTATGTGATTGCTGAGAGA-3' and BF2:

5'DATCCCTAAGATTTTATTATCCCAAGTA-3' (Nedayefan co. Iran) was used for polymerase chain reaction (PCR).

DNA amplifications were performed in 20 μ L volumes that contained 10 to 100 ng of DNA, 0.5 μ M of each primer, in the presence of 2 mM MgCl₂, 100 μ M each of dNTP, 50 μ M KCl, 20 mM Tris-HCl, pH 8.4, and 2.5 U recombinant DNA polymerase (Jena Bioscience Co., Germany). Amplification was performed in a DNA thermal cycler (Gradient Eppendorf) programmed for 94°C (seven minutes) as the initial denaturation step, followed by 35 cycles at 94°C (35seconds), 52°C (50 seconds), 72°C (55 seconds), and then 72°C for five minutes.

PCR products were electrophoresis in 1.2% agarose gel, and after staining with 0.5 μ g/mL ethidium bromide they were visualized under ultraviolet (UV) light. The determination size of fragments was compared with 100 bp DNA ladder-size markers (Jena Bioscience co. Germany) (22).

4. Results

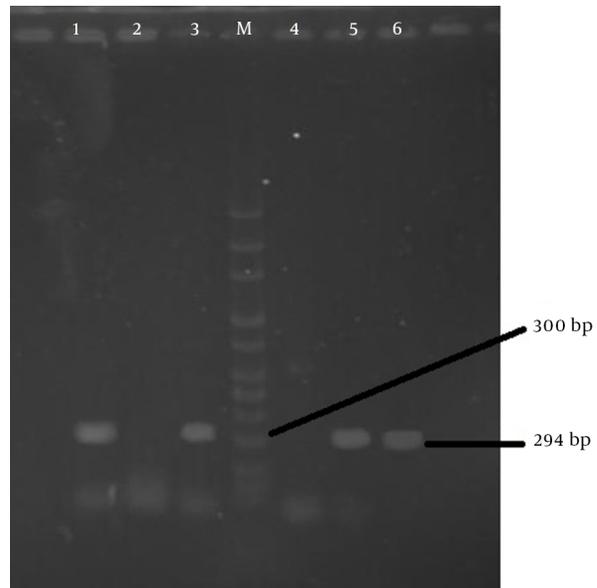
In this study 100 diarrheal fecal samples of 55 males and 45 females obtained from outpatients and hospitalized children under the age of 5 were processed. Of these, 59% of the specimens were positive, of which 49 (83%) had one pathogen, nine (15.3%) had 2 pathogens, and one (1.7%) had 3 pathogens. Of 70 isolated pathogens, the most detected pathogen was rotaviruses 40 (57.2%), followed by adenovirus 13 (18.6%) and only 10 (14.3%) *B. fragilis* were isolated, four (5.72%) of which were enterotoxigenic (Table 1 and Figure 1).

Table 1. Distribution of 70 Pathogens Detected from 59% Positive Specimens

Pathogens	No. (%)
<i>Rotavirus</i>	40 (57.2)
<i>Adenovirus</i>	13 (18.6)
<i>Bacteroides fragilis</i>	10 (14.30)
ETBF	4 (5.72)
Non-ETBF	6 (8.58)
<i>Giardia lamblia</i>	3 (4.28)
<i>E. coli</i> spp.	2 (2.85)
<i>Shigella</i> spp.	2 (2.85)
<i>Salmonella</i> spp.	0
Total	70 (100)

Bacteroides fragilis was isolated from children with a mean age of 2.3 years (range, 3 months to 5 years). All isolates were susceptible to MTZ, while only 10% were susceptible to clindamycin. Clinical characteristics of patients

Figure 1. ETBF Gene PCR Product Detected by Agarose Gel Electrophoresis (1.2%)



M (100 - 3000 bp DNA size marker), lanes 1, 3, 5 and 6 are clinical isolates of positive ETBF gene.

with positive culture for *B. fragilis* and MIC for metronidazole (0.38 - 1 μ g/mL) and clindamycin (2 - 256 μ g/mL) are shown in Table 2.

5. Discussion

Bacteroides fragilis usually constitutes 1% to 2% of the normal human gastrointestinal microbial flora (23). This important opportunistic obligate anaerobic pathogen commonly causes human extra-intestinal polymicrobial infections (17). Recently, the association of *B. fragilis* as etiological pathogen of gastrointestinal disease has been highlighted by some researchers (23). This opportunistic pathogen causes diarrheal disease via production of zinc metalloprotease enterotoxin (11).

In this study, all isolated *B. fragilis* studied using the PCR technique and ETBF were discovered in four (4%) cases. These findings are similar to the results of other studies carried out by Albert et al. and Jiang et al., who reported 3.5% and 4% ETBF prevalence among children with diarrheal disease (24, 25). However these results are in contrast with the results reported by Durmaz et al. in Turkey (11%) and by Niyogi et al. in India (2.3%) (26, 27).

Although some studies show that regardless of being diarrheal disease, one may be an ETBF carrier (23), Sack et al. reported 4% ETBF as an important etiologic agent in acute

Table 2. Clinical Characteristics of Patients and MIC Obtained for *B. fragilis* Isolates

Isolate	Age	Sex	MIC ($\mu\text{g/mL}$), Obtained for		Enterotoxin Production	Hospitalization
			MTZ	CD		
1	1-2	F	0.50, S	2, S	Negative	In-patient
2	2-3	F	0.50, S	256, R	Negative	In-patient
3	4-5	M	0.50, S	256, R	Negative	Out-patient
4	4-5	F	1, S	256, R	Negative	Out-patient
5	0-1	F	0.75, S	256, R	Positive	In-patient
6	1-2	M	0.75, S	256, R	Positive	In-patient
7	2-3	M	0.38, S	256, R	Positive	Out-patient
8	0-1	M	0.38, S	256, R	Negative	Out-patient
9	3-4	M	0.50, S	256, R	Negative	Out-patient
10	0-1	F	0.50, S	256, R	Positive	Out-patient

Abbreviations: CD, clindamycin; F, female; M, male; MIC, minimum inhibitory concentration; MTZ, metronidazole; R, resistant; S, sensitive.

diarrhea in children older than 1 year in a controlled study (9).

Due to the decrease in sensitivity to antibiotics among anaerobic bacteria, selection of antibiotic drugs for treatment of infections caused by these bacteria has been difficult (23). Metronidazole has been the drug of choice for the treatment of infection caused by anaerobic bacteria worldwide for nearly 40 years but the recent emergence of metronidazole resistance is a matter of great concern (17). In our study, 100% of isolated *B. fragilis* were susceptible to metronidazole. These results are similar to others study carried by Nakano et al. (16). In contrast to the results obtained in our study, Akhi et al. reported 5% resistance to metronidazole among *B. fragilis* isolates (7). In other previous studies, metronidazole resistant *Bacteroides* spp. has been reported up to 15% (28). Nakano et al. also reported 34.2% resistance to metronidazole (16). Thus, quick recognition of metronidazole-resistant strains is vital for early initiation of correct antimicrobial therapy and for limiting the unsuitable administration of antibacterial drugs.

Recently there has been an increase in the resistance of bacteria to certain antibiotics such as clindamycin. In accordance with the results reported by Akhi et al. (7), we isolated 90% resistance to clindamycin. A contrasting study carried out by Seifert and Dalhoff reported 22.7% resistance to clindamycin in Germany (29). Trevino et al. also reported 45% clindamycin resistant *B. fragilis* in Spain (30). Thus, information regarding the prevalence of resistance among *B. fragilis* in each geographical region is important for the appropriate administration of antibiotics (14, 31). With the rapid increase in the frequency of clindamycin resistance in *B. fragilis*, this drug is no longer considered a

first-line treatment for infections caused by this organism (31).

In our study, rotavirus was the commonest agent that was identified as a pathogen in cases of diarrhea, which is in agreement with other studies carried out by Vu Nguyen et al. Rotaviruses continue to be the main cause of gastroenteritis in children in industrialized areas and developing countries. The virus is an important cause of hospitalization and constant disease in children living in the U.S. and cause of death in developing countries (6).

The results obtained in this study show ETBF isolate from children with diarrheal disease in our hospitals. Metronidazole is the drug of choice for treatment of these infections, but clindamycin is an inappropriate option for empirical antibiotic therapy. Considering the increased resistance to other antibiotics, particularly metronidazole as reported by other studies, highlights the importance of determining antibiotic susceptibility patterns for appropriate antibiotic therapy. A close relationship between physicians and microbiology laboratories is essential to achieving these aims.

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commission of Tabriz University of Medical Sciences approved this study (Number: 5/4/9094-28th Jan. 2014).

Footnotes

Authors' Contribution: All authors have contributed significantly to this work. Mohammad Taghi Akhi: Supervisor, bacteriologist. Sirius Jedari Seifi: Supervisor, virologist; Mohammad Asgharzadeh: Molecular biologist; Mohammad Ahangarzadeh Rezaee: Bacteriologist, sample collection; Shahram Abdoli Oskuei: Specialist in infectious diseases of children, introducing patients; Tahereh Pirzadeh, Mohammad Yousef Memar, and Naser Alizadeh: Cooperation in performance of practical work; Hasan Seifi Yarijan Sofla: Taking part in all stages of research.

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