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Comparison of E test and Disk Diffusion Test for Antibiotic Resistance Testing of Enterotoxigenic and Non-Enterotoxigenic *Bacteroides fragilis* Isolated From Stools

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Background: Enterotoxigenic *Bacteroides fragilis* (ETBF) are one of the most important anaerobic bacteria which cause diarrhea in human beings. A bft gene is coded for enterotoxin production called fragilysin. Most of them have acquired resistance to antianaerobic bacteria agent like other facultative anaerobic bacteria. E test and different dilution methods are usually performed for antimicrobial susceptibility determination of *B. fragilis*.

Objectives: The aims of this study are to recognize ETBF by PCR method and also to evaluate efficiency the disk diffusion method (DDM) in comparison with the E tests for antimicrobial susceptibility of *B. fragilis* isolates.

Materials and Methods: bft gene was detected among 157 *B. fragilis* isolated from patients and healthy individuals by PCR. Antimicrobial susceptibility of all isolates was determined by DDM and E tests methods.

Results: Nineteen (12.1%) *B. fragilis* containing bft gene from diarrheic (n = 14) and nondiarrheic (n = 5) feces were detected among 157 *B. fragilis* isolates. The highest resistance for ciprofloxacin, cefotaxim, cefoxitin with DDM and E test were 100%, 60%, and 65% respectively, while the lowest resistance in two methods was obtained for imipenem, piperacillin / tazobactam, and metronidazol. 100% agreement for some of antibiotics such as imipenem ($P \leq 0.05$), and no correlation for others were observed among the antimicrobial susceptibility results obtained by the two methods ($P \geq 0.05$).

Conclusions: The presence of bft gene in *B. fragilis* isolates would not certainly result in diarrhea among patients. There is not enough accordance between DDM and Swedish E tests for antimicrobial susceptibility of *B. fragilis* for some antibiotics, although in other cases a good agreement was observed.

Keywords: Bacteroides fragilis; Antibiotic Resistance; Disk Diffusion Antimicrobial Tests

1. Background

Bacteroides fragilis is one of the most important anaerobic non-spore-forming bacteria among human body's anaero-biomicrofloras. This bacterium has an important role in causing human's diarrhea and other infections such as post surgery infections, septicemia, gynecological, skin and soft tissue infections, brain abscess and meningitis (1). Enterotoxin produced by *B. fragilis* is an important factor resulting in diarrhea which is controlled by bft gene. Enterotoxin or fragilisin is a zinc-dependent meta-protease with molecular weight of 20000 Da. This toxin causes the intestinal cells to lose fluids by removing epithelial barriers (2).

An assay using HT291C1 cells facilitated the detection of this toxin in research laboratories, but PCR assay is a valuable technique for detecting DNA sequences specific to organisms or for individual genes (3). In general, PCR assays are more objective than cell culture assays for detecting toxins. *B. fragilis* such as facultative anaerobic bacteria is capable of gaining resistance against different antibiotics, and in this case it takes the first place among anaerobic bacteria (4). Determining resistance pattern of these bacteria is completely important for treating resulted infections, and is usually conducted by agar dilution or E test method based on guidelines of CLSI (5, 6).

Disk diffusion method (DDM) is based on the inhibited growth zones, when fixed concentrations of an antimicrobial compound diffuse from the antibiotic disks into

Implication for health policy/practice/research/medical education:
B. fragilis isolated from patients with diarrhea contain bft gene, but its presence in different isolates would not certainly result in diarrhea among patients. In addition the results of DDM for anaerobes even for fast growing anaerobe bacteria such as *B. fragilis* should be confirmed by other reliable ones like Swedish E test or agar dilution methods.

agar plate, which has formerly been inoculated with the test organism. The inhibition zones around the disks are measured after suitable incubation and interpreted as sensitive, intermediate, and resistant zones (7, 8). The epsilometer test (E-test) is the combination of the diffusion and the ability to establish a minimum inhibitory concentration (MIC) or breakpoint result. It is based on diffusion of a preformed antimicrobial gradient from coated plastic strips onto an agar plate inoculated with the test organism (9, 10).

DDM is not approved by CLSI for this bacterium and criteria for result interpretation are not determined yet. Since agar dilution test is a time consuming and expensive one, most of researchers are making efforts to standardize disk diffusion method for evaluating anaerobic bacteria's antibiotic sensitivity such as that of the *B. fragilis*. Barry et al. evaluated sensitivity of *B. fragilis* to nine antibiotics by disk diffusion method in 1990 and reported collected results as satisfactory (11). However, other researchers claimed this method as an absolutely improper method for evaluating anaerobic sensitivity (12).

2. Objectives

The objectives of this research are to recognize enterotoxigenic *B. fragilis* by PCR method, and also to evaluate efficiency thedisk diffusion method in comparison with the E tests method for antimicrobial susceptibility testing of *B. fragilis* isolates.

3. Materials and Methods

One hundred fifty seven *B. fragilis* strains were isolated from 188 diarrheic and non-diarrheic fecal samples of patients and healthy individuals, and identified by biochemical techniques as described before (13, 14). Enterotoxigenic *Bacteroid fragilis* (ETBF) strains were detected by bft gene detection. DNA extraction- to detect the bft gene, one colony of each isolate grown in reduced blood agar under anaerobic conditions (CO2 = 10%, H2 = 10%, N2 = 80%) was suspended in 300 µl of ultrapure water, homogenized, boiled for 20 min and centrifuged at 14 000g, for 10 min. Supernatants were used as templates in PCR reaction performed with following specific primers for amplification of 294 bp fragment (BF1:5'dGACGGTGTATG TGATTTGTCTGAGAGA-3'and BF2: 5'dATCCCTAAGATTTTAT-TATCCCAAGTA-3') (15, 16).

DNA amplifications were performed in 25 µl containing 2.5 µl of 10 X PCR buffer, 1.25 µl of MgCl2 (1.5 mM), 2.0 µl of dNTP mixture (0.2 mM), 0.25 µl of Taq DNA polymerase (0.5 U), 1 µl of each primer (0.4 mM), 7 µl of ultrapure water, and 10 µl of DNA template (Concentration of DNA was determined by measuring the ratio of OD values at 260/280nm). Amplification was performed in a DNA thermal cycler (Gradient Eppendrof) 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% 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 100 bp DNA Ladder was used as a molecular mass marker. Gels were stained with ethidium bromide (0.5 µg ml-1) and photographed on a gel documentation system (UVP, USA) for the analysis of bands (15).

3.1. Susceptibility Tests

In disk diffusion technique, the zone inhibition size of antibiotic disks is mostly dependent on antimicrobial concentration, a critical population of organisms and time. These conditions make it unsuitable for testing slow growing organisms. However, anaerobes that grow well within 24 h seem to be tested by disk diffusion as a screen for resistance (17). In this study we performed sensitivity test for *B. fragilis* isolates based on a modified disc diffusion method for susceptibility testing of fastidious bacteria according to the CLSI guidelines. Since some of anaerobic bacteria do not grow well enough on Muller-Hinton to permit antimicrobial susceptibility testing, so we decided to use brucella blood agar supplemented with hemin and vitamin K (BBA) for antimicrobial susceptibility testing of *B. fragilis* by E test and disc diffusion methods (17).

B. fragilis isolates were subcultured on to Columbia agar (Oxoid, UK), containing 5% (v/v) sheep blood, and were incubated under anaerobic conditions (CO 2 = 10%, H 2 = 10%, N 2 = 80%) at 37°C for 48 h. Following incubation, suspensions of pure cultures were prepared in sterile physiological serum, and were adjusted to give inoculums with an equivalent cell density to 0.5 McFarland turbidity standards (1.5×108 cfu/mL). Cell suspensions were then swabbed evenly onto BBA plates. On each 9 cm plate containing BBA, E test gradient strip (AB biomeriux, Sweden) of following antibiotics and corresponding disc (Mast Ltd) were applied: amoxicillin/clavulanic acid, imipenem, piperacillin/tazobactam, metronidazol, cefoxitin, cefotaxim, ciprofloxacin, clindamicin, chloramphenicol, rifampin (antibiotic concentration of E tests and discs are shown in Table 1).

Plates were incubated under anaerobic conditions at 37°C for 48 h. The inhibition zones for each antibiotic disk were measured by ruler and were interpreted according to the guidelines recommended for fastidious bacteria by CLSI (6, 18). The MIC values were read where the edge of the inhibition ellipse intersected the strip. Readings for each culture were recorded and classified as being resistant or sensitive based on their MIC breakpoints according to the manufacturer's and the CLSI instructions (6). Although we already know that all anaerobic bacteria are resistant to aminoglycosids, to confirm anaerobic origin of isolates we tested gentamicin too.

Antimicrobial resistance of the all *B. fragilis* isolates to

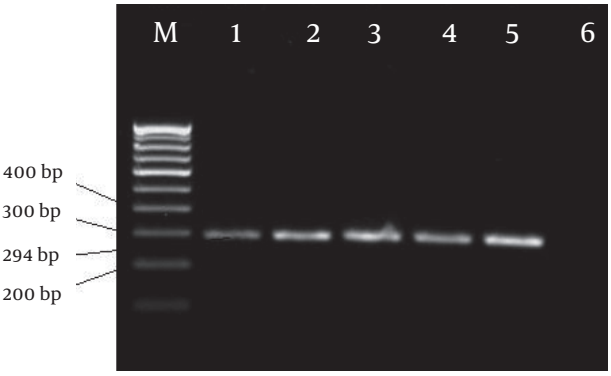
each of the 11 antimicrobials, using both the agar disk diffusion and E-test methodologies was compared by Fischer exact two-tailed analysis with significance defined at the 95% level ($P \leq 0.05$). All statistical analyses were performed using SPSS version 18 software.

4. Results

In this study 157 *B. fragilis* isolates from diarrheic (n = 32, 20.38%) and non-diarrheic (n=125, 9.62%) fecal samples were evaluated for bft gene by PCR. Nineteen (12.1%) *B. fragilis* containing bft gene from diarrheic (n = 14) and non-diarrheic (n = 5) were detected (Figure 1). While there was 100% agreement for some of antibiotics such as imipenem, ciprofloxacin, rifampin among the results obtained by disk diffusion and E tests ($P \leq 0.05$), no correlation for others were also observed among the results of two methods ($P \geq 0.05$) (Table 1).

The lowest sensitivity in two methods was detected for ciprofloxacin (0% in E test, and 0% in DDA). The highest resistance for ciprofloxacin, cefotaxim, cefoxitin with DDA and E test were 100%, 60%, and 65% respectively, while the lowest resistance in two methods was obtained for imipenem, rifampin, piperacillin/tazobactam, and metronidazol (Table 1).

Figure 1. PCR Amplification of bft Gene of Enterotoxigenic *B. fragilis*



M; 100 bp DNA ladder, 6; negative control, 1, 2, 3, 4, 5 are representative of enterotoxigenic *B. fragilis* isolates (294 bp).

Table 1. Susceptibility of *Bacteroid fragilis* Isolated From Diarrheal and non- Diarrheal Patients and Healthy Individuals Stools by E Test and Disk Diffusion Methods

Antibiotics	Antibiotic Content of E tests Strips, μg/mL) (disks, μg)	ObtainedMIC by E tests, μg/mL	Obtained Results by E tests		Obtained Inhibition Zone by Disk Diffusion for Different Isolates, mm	Obtained Results by Disk Diffusion		P value
			S %	R %		S %	R %	
Amoxicillin/Clavulanic acid	0.016 - 256(20/10)	0.5 - 32	35	65	17 - 28	75	25	0.07
Imipenem	0.002 - 32(10)	0.3 - 1.1	100	0	32.4	100	0	0.001
Piperacillin/ Tazobactam	0.016 - 256(100/10)	0.01-128	95	5	32.4	100	0	0.04
Metronidazol	0.016 - 256(5)	0.2 - 64	95	5	15 - 30.2	93	7	0.03
Cefoxitin	0.016 - 256(30)	≤ 64	0	100	21 - 29	35	65	0.65
Cefotaxim	0.016 - 256(30)	≤ 64	0	100	18 - 32	40	60	0.75
Ciprofloxacin	0.002 - 32(5)	≤ 32	0	100	17	0	100	0.001
Clindamicin	0.016 - 256(2)	2-16	10	90	14 - 25	38	62	0.65
Chloramphenicol	0.016 - 256(30)	2-64	45	55	24 - 30.2	65	45	0.55
Rifampin	0.016 - 2569(5)	0.03-0.05	100	0	20 - 26	100	0	0.001
Gentamicin[a]	0.016 - 256(10)	≤ 128	0	100	2	0	100	0.001

[a] Anaerobic are genetically resistant to aminoglycosides

5. Discussion

B. fragilis has been known as a cause for diarrhea in animals and humans. Although they usually form 1-2% of the normal human intestinal flora, they may play role in extra intestinal infections. Recently ETBF isolation from healthy children and adults' feces and also from those with diarrhea was reported (14). Researchers have discovered *B. fragilis* enterotoxin directly from a part of feces sample whose culture hadpositive results for ETBF (14) and different studies in Brazil reported 1.5-3% ETBF among stool samples (19). In other studies high numbers of ETBF have been reported in different points of the world (3, 20), for instance Kato et al.(1996) reported a percentage

of 18.5% in Japan, while it was about 14% in Poland and Holland (21).

Other researchers have reported isolation percentage of ETBF as 6-12% in the USA, 11% in Italy, 12% in Sweden, and 2-6% in Bangladesh (15). Also studies showed that regardless of being diarrheal or non-diarrheal one can host ETBF organisms as carrier and natural flora of intestine (15). In this study, totally 157 *B. fragilis* including 32 *B. fragilis* of diarrheal feces and 125 cases of non-diarrheal feces were studied by PCR to find out the bft gene. Nineteen cases (12.1%) including bft gene were discovered in diarrheal feces (14 cases) and non-diarrheal feces (5 cases). These results are in accordance with many obtained results in the world (15), however are in contrast with the results reported in Brazil which are significantly lower than this number (19).

Due to lack of scientific information about occurrence of ETBF among Tabriz population, it is not possible to compare the findings of this research with previously obtained results. Observation of ETBF among *B. fragilis* islolates from feces samples of non-diarrheal persons could prove the presence of healthy carriers as infection resources for susceptible people. It is probable that no toxin would be produced in spite of presence of bft gene, due to environmental factors.

Previously for routinely curing infections caused by anaerobic bacteria, physicians were selecting antimicrobial materials based on experiences on that region's medical sciences, but nowadays selection of antimicrobial materials has been difficult due to the increase in drug resistance among facultative and obligate anaerobic bacteria (4). As a result it is inevitable to perform tests of bacteria sensitivity to antibiotics in in-vitro method.

Many approaches were invented for this goal, among which the disk diffusion method is acceptable among most of microbiologists due to its high speed and ease of performance, and its accessibility. But it seems that this method lacks acceptable quality in anaerobic cases (4). In the second part of this study, the more practical and inexpensive disk diffusion method, which is suitable for the analysis of a small number of isolates, as normally occurs in clinical laboratories, was evaluated as an alternative to the E test to discriminate between susceptible and resistant isolates of *Bacteroides fragilis*. Although this method has not yet been standardized for *B. fragilis*, the results of different studies on facultative bacteria have shown that there is a good correlation of the disk diffusion method with E-test. For example various researches conducted such a comparison between these two methods on different bacteria such as *Helicobacter pylori* (22), *Campylobacter* spp. (23), *Staphylococcus aureus* (24), coagulase-negative staphylococci (25), *Stenotrophomonas maltophilia* (26), and *Escherichia coli* (27).

Disk diffusion method has also been conducted on some fast-growing anaerobic bacteria and susceptibility criteria for some antibiotics have been provided (18). In the research that Barry, *et al*. performed on anaerobic bacteria, disk diffusion method was reported to be appropriate and useful for fast growing anaerobic bacteria such as *B. fragilis* (11). Other researches performed this method using vancomycin and metronidazole disks on *C. difficile* isolates and observed a good agreement with E tests results (28). Dubois and Pechere have reached a strong correlation between the results of the disc diffusion for anaerobic bacteria in comparison with results reported by agar dilution method using cephalothin, cefamandole, cefazolin, and cefoxitin antibiotic discs. But the results were not satisfactory for cephalothin against *B. fragilis* (29). On the other hand in the study of Tally et al., the assessment of anaerobic sensitivity toward antibiotics by disc diffusion method was not declared satisfactory (12).

In 2001, the conditions to use the disk diffusion test for fast growing anaerobic such as *B. fragilis* and also detailed criteria for certain antibiotics such as penicillin, clindamycin and metronidazole were reported by King et al. (18). In this study results of sensitivity tests with agar disk diffusion in comparison with E test on *B. fragilis* isolated from patients and healthy people were reported. The results showed significant correlation between these two methods for some antibiotics such as imipenem, piperacillin/tazobactam, metronidazol, ciprofloxacin, chloramphenicol, rifampin and gentamicin; while, there was no significant correlation in others (Table 1). The obtained results for anaerobes by disk diffusion method indicated that this method is not satisfactory for all antibiotics.

We hence concluded that *B. fragilis* isolated from patients with diarrhea contain bft gene, but its presence in different isolates would not certainly result in diarrhea among patients. There is not enough accordance between DDM and Swedish E test susceptibility results of *B. fragilis* for some antibiotics, although in other cases a good agreement was observed. Therefore the results of DDM for anaerobes even for fast growing anaerobe bacteria such as *B. fragilis* should be confirmed by other reliable ones like Swedish E test or agar dilution methods.

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Authors' Contribution

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