Determination of Antibiotic Sensitivity of *Bacteroid fragilis* Isolated from Patients and Healthy Individuals in Imam Reza Center of Medical Teaching and Treatment-Tabriz

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Background: *Bacteroides fragilis* are among the most important anaerobic bacteria behind most of the anaerobic infections. They have acquired resistance to essential treatment antibiotics of anaerobic infections, more than other anaerobic bacteria.

Objectives: The goal of this study is to determine the resistance of isolated *B. fragilis* against common anaerobic infections treatment antibiotics.

Patients and Methods: A total of 188 fecal samples including 59 samples from hospitalized patients, 84 samples from outpatients, and 45 samples from healthy individuals were collected. The samples were cultured in Bacteroides- Bile- Esculine agar and Kanamycin-Vancomycin-Laked Blood media and were incubated in anaerobic atmosphere at 37°C for at least 48 hours. Suspected one millimeter sized colonies with black surroundings, were selected and further studied using MID8, as well as biochemical tests. For MIC determination of antibiotics against isolated *B. fragilis*, Etest was used.

Results: There wasn't any difference between antibiotic resistance patterns of isolated *B. fragilis* from hospitalized patients or outpatients, including diarrheal and non-diarrheal cases, and resistant pattern of isolates from healthy individuals. All or most of isolated *B. fragilis* were susceptible to imipenem (100%), metronidazole (95%), rifampin (100%) and piperacillin/tazobactam (95%). On the contrary, they exhibited resistance to other antibiotics such as clindamycin (90%), and chloramphenicol (55%).

Conclusions: In the present study, we have figured out that a number of the important anaerobic infections treatment antibiotics have lost partly or totally their effectiveness on *B. fragilis*.

Keywords: Bacteroides fragilis; Antibiotic Resistance; Anaerobic Infection; Metronidazol; Imipenem

1. Background

Bacteroides are among the anaerobic Gram-negative bacilli and present in the natural flora in different human body parts such as oral mucosa, oropharynx, gastrointestinal tract (gut volume), and female genital (1). More than fifty species of this genus have been identified, the most important of which is the fragilis species. *Bacteroides fragilis* group is considered the main anaerobic bacteria causing infections in human beings. Despite of its protective role, it creates various infections in different anatomical sites of humans, such as abdominal cavity, pelvis, liver and soft tissues. Most of these infections, as well as some other infections in the lungs, brain and septicemia are caused by the patient's natural flora, especially colon flora. It is the causing agent in one third of anaerobic infections (1, 2). The role of this bac-

terium in causing gastrointestinal infections (diarrhea) in animals and humans, and the relationship between enterotoxins and diarrhea in human have been proved by researchers (3).

In researches that have been conducted recently, have identified some strains resistant to metronidazole as well as other antibiotics such as ampicillin, ampicillin/sulbactam, piperacillin / tazobactam, meropenem, ceftriaxone, clindamycin and chloramphenicol (4, 5). *B. fragilis* strains resistant to penicillin and cephalosporin are significant due to their beta-lactamase production, and it is the first anaerobic bacterium that produces beta-lactamase (1, 4). But some special beta-lactams such as cefoxitin and imipenem are resistant toward the beta-lactamase produced by these bacteria and could be used in therapy. Most of the penicillin, when mixed with a

beta-lactamase inhibitor such as clavulanic acid, become efficient against more than 95 percent of beta-lactamase producing strains (4, 6). Among *B. fragilis* strains, 5% to 15% of them are resistant to clindamycin; whereas this is noticed in more than 30% of other species of *B.* group(6). In another study carried out in Turky, 33-43% resistance rate of *B. fragilis* to clindamycin was reported (7). Fluoroqinolones are also used in combination with clindamycin and metronidazole for the treatment of *B. fragilis* infections. Recently increased resistance to most of Fluoroqinolones have been observed but newly synthesized Fluoroqinolones including clinafloxcin, sintafoxacin and garenoxacin are generally more active against *Bacteroides* species (8).

Up to the recent years, it was thought that the sensitivity of this bacterium to antibiotics is constant, so sensitivity determination was not done for it routinely (1). In view of this increased resistance CLSI (Clinical and Laboratory Standards Institute) considers periodical determination of sensitivity for *B. fragilis* in any area as a necessary task since there are differences among the isolates of various areas.

2. Objectives

Considering lack of any information about culture, isolation, and prevalence rate of *B. fragilis* in feces and sensitivity pattern of these bacteria in Tabriz, finding answers to the above mentioned subjects are among the goals of this study.

3. Patients and Methods

3.1. Sampling

In a descriptive study, any fecal sample (outpatient or hospitalized, including diarrheal and non-diarrheal cases), that consecutively was sent to laboratory of Imam Reza Educational and Medical Center by physicians for culturing and determining etiological agent was selected as a sample for this project. All samples were transferred to anaerobic laboratory in the Faculty of Medicine immediately. Total of 188 fecal samples were collected, where 59 samples of which were of hospitalized patients, 84 samples of outpatients, and 45 samples of healthy people.

Fecal samples of healthy people were collected from people who had no diarrhea or any other gastrointestinal disorder and were presented to the laboratory for other medical reasons. These healthy people were justified orally or in some cases were acquired written consent. Sampling method of this project was simple random and all samples were accepted except those who had consumed antibiotics during the week before.

3.2. Bacteroides fragilis Culturing and Identification Methods.

Samples have been cultured in Bacteroides Bile Esculine

agar (BBE, Himedia Laboratories Pvt. Ltd, India) and Kanamycin-Vancomycin-Laked Blood (KVLB, Basal Medium is Brucella agar; (Fluka Chmie AG CH-9471 Buchs, Switzerland) media, and subsequently incubated for 48 hours at 37°C in anaerobic atmosphere (H$_2$ =10%, N$_2$ = 80%, CO$_2$ = 10%) using Anoxomat machine and Mart jar (MART Microbiology B.V. The Netherlands). Suspicious one millimeter colonies with black surroundings (showing esculine hydrolyze) were selected in BBE medium, and further identified after conducting anaerobic tolerance test using MID8 (Mast Identification 8, according to manufacturer company's instructions; Table 1) and also some biochemical tests such as catalase production, indole, and sugar fermentation (sucrose, arabinose, xylose, and rhamnose) (5 , 9). MID 8 consists of a circle, around which there are six disks of each of the following antibiotics: Erythromycin (60 µg), Rifampin (15 µg RP), Colistin sulfate (10 µg CO), Penicillin G (2 PG Units), Kanamycin (1000 µg K) And Vancomycin (5 µg VA). *B. fragilis* were identified using Table 1, which is provided by the manufacturing company, and other tests.

3.3. Sensitivity to Antibiotics Evaluation Test

In order to determine resistance pattern and Minimum inhibitory concentration (MIC) of common antibiotics to *B. fragilis* isolated from samples, Etest (AB biomeriux, Sweden) was used. Minimum inhibitory concentration was determined on columbia agar (Himedia Laboratories Pvt. Ltd, India) containing 5% sheep blood for imipenem, metronidazole, piperacillin/tazobactam, clindamycin, chloramphenicol, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, ciprofloxacin and rifampin antibiotics. All plates were incubated anaerobically for 24 hours at 37°C. The producer's instructions and the guidelines of Clinical and Laboratory Standards Institute (CLSI) were considered in testing and reading the obtained results (10). Data were analyzed by SPSS software, version 16, using χ^2 or Fisher's exact tests. A p-value of < 0.05 was considered statistically significant.

4. Results

Of 188 fecal samples, total of 157 *B. fragilis* were isolated from outpatients (73 cases), hospitalized patients (51 cases) including diarrheal and non-diarrheal cases, and healthy people (33 cases) (Table 2). Unlike the occurrence of *B. fragilis* isolation between patients and healthy individuals (P value ≥ 0.05), the occurrence of *B. fragilis* isolation among diarrheal and non-diarrheal stool samples was significantly meaningful (P value ≤ 0.02). Antibiotic resistance pattern of patients had no difference with respect to that of healthy people (P value ≥ 0.05). Isolated *B. fragilis* were sensitive to imipenem (100%), metronidazole (95%), piperacillin/tazobactam (95%), and showed resistance against clindamycin (90%), chloramphenicol (55%) and some of those antibiotics that were previously used as effective medications of anaerobic infections.

Table 1. Interpretation Criteria for *B. fragilis* Identification (Provided by MID 8 Manufacturer)

Tests Organisms	Antimicrobial					
	E [a]	RP [a]	CO [a]	PG [a]	K [a]	VA [a]
***B.fragilis group* ATCC**	S	S	R	R	R	R
25285	S	S	S*	S*	R	R
Prevotellamelaninogenica/oralis	S	S	R	S*	R*	S*
Porphyromonasspp.	S	S	R	S*	R*	S*
***B.ureolyticus* ATCC 33387**	S	V	S	S	S	R
Fusobacteriummortiferum/varium	R	R	S	S	S	R
***F.varieum* ATCC 27725**	R*	V	S	S	S	
Other*fusobacterium*						
Gram positive cocci	S	S	R	S*	V	S
Clostridium* spp. e.g. *Clostridium	S	S	R	S*	V	S
***perfrin genes* ATCC 13124**						
Gram positive bacilli (NSGPG)	S	S*	R	S*	V	S
Gram negative cocci	S	S	S	S	S	R

[a] Abbreviations: S, Sensitive; S*, Majority sensitive; E, Erythromycin; R, Resistant; R*, Majority resistant; RP, Rifampin; V, Variable; NSGPG, Non-Sporing ; CO, Colistin sulphate; PG, Penicillin G; K, Kanamycin; VA, Vancomycin

All isolated *B. fragilis* were also sensitive to rifampin, i.e. the antibiotic which is also used as *Bacteroides* identifying test. MIC of determined antibiotics to isolated colonies is shown in Table 3.

Table 2. Frequency of *B. fragilis* Isolates From Stool Samples of Inpatients, Outpatients and Healthy Individuals

Patients	Total Samples, No. (%)	Isolated *B. fragilis* No. (%)	Diarrheal Stools /*B. fragilis* Isolated, No. (%)	Non-Diarrheal Stools/*B. fragilis* Isolated, No. (%)
Inpatients	59 (31.34)	51 (32.48)	24/20 (83.34)	35/31 (88.57)
Outpatients				
Healthy	84 (44.46)	73 (46.5)	14/12 (85.71)	70/61 (87.14)
Individuals	45 (24)	33 (21.02)	--	45/33 (73.34)
Total	188 (100)	157 (100%)	38/32 (84.21)	150/125 (83/34)

Table 3. MIC of Different Antibiotics to *B. fragilis* Isolated From Diarrheal and Non-Diarrheal Patients Stool in Outpatient, Inpatient and Healthy Individuals

Antibiotics	Antibiotic Content of E-tests, μg/mL	MIC, Obtained by E-tests, μg/mL	CLSI Interpretative MIC, μg/mL			Obtained Results Among Isolated *B. fragilis*	
			S ≤	I	R ≥	S %	R%
Amoxicillin/Clavulanic acid	0.016-256	0.5-32	4	8	16	35	65
Imipenem	0.002-32	0.3-1.1	4	8	16	100	0
Piperacillin/Tazobactam	0.016-256	0.01-128	32	64	128	95	5
Metronidazol	0.016-256	0.2-64	4	16	32	95	5
Cefoxitin	0.016-256	64 ≥	16	32	64	0	5
Cefotaxim	0.016-256	64 ≥	16	3	64	0	100
Ciprofloxacin	0.002-32	32 ≥	0.06	0.1-0.5	1	0	100
Clindamicin	0.016-256	2-16	2	4	8	10	90
Cloramphenicol	0.016-256	2-64	8	16	32	45	55
Rifampin	0.016-256	0.03-0.05	1	2	4	100	0
Gentamicin [a]	0.016-256	128 ≥	-	-	-	0	100

[a] Anaerobic are geneticaly resistant to aminoglycosides

5. Discussion

The importance of anaerobic bacteria in the etiology of human gastrointestinal infections is well known. Among these microorganisms, the ten related species of Gram-negative rods of the *B. fragilis* group stand out (11 , 12). In developing countries, these diseases are some of the most important causes of morbidly and especially mortality, in children of low age (13). In the present study, results show that the frequency of *B. fragilis* isolation among diarrheal and non-diarrheal stool samples were significantly meaningful (P value ≤ 0.02), indicating possible role of *B. fragilis* in producing diarrhea in patients (Table 2). The involvement of *B. fragilis* as etiological agents of gastrointestinal disease has been highlighted by several scientists in recent years (14).

There are many reports on antibiotic resistance increase of anaerobic bacteria especially on *B. fragilis*. Unnecessary and high usage of some antibiotics possibly causes selective pressure and spread of antibiotic resistant strains. It is clear that antibiotic resistance level could vary from one geographical point to another. These differences in antibiotic resistance even can vary from one hospital to another, which shows the usage of an especial antimicrobial material in that place (4). There is a lack of information on anaerobic sensitivity pattern in Tabriz, especially about *B. fragilis* which is an important anaerobic bacterium. For this reason, fecal samples were collected on winter of 2011 to provide the possibility of conducting sensitivity determination tests of isolated *B. fragilis* against various antibiotics.

The observation of these bacteria's resistance level toward some antimicrobial materials and their sensitivity to antibiotics such as metronidazole and imipenem in recent years, would significantly help in clinical therapy of infections suspicious of anaerobic like infections caused by *Bacteroides*. The results of current research showed isolated *B. fragilis* are sensitive to metronidazole and imipenem, which is in accordance with the findings of some researchers (12, 13). However, totally resistant strains obtained from animals and human against imipenem (15, 16), and strains resistant to metronidazole were also found in different studies (17, 18).

Information collected from this research shows that there is no significant difference in the results of sensitivity pattern of collected *Bacteroides* from diarrheal and non-diarrheal feces of all patients and even from healthy persons. In a research conducted by Ulger et al in Turkey (7), resistance pattern of the ones isolated from feces and clinical infections is in accordance with our study.

Since clindamycin has been used as an effective antibiotic for a long term, the resistance against this drug has been reported from different countries (6, 17). In this research, increased resistance of isolated *B. fragilis* to clindamycin is obvious which shows accordance with reported results of other researchers (6, 17). Clindamycin resistance has been shown to be acquired by macrolide-lincosamine streptogramin B - resistance determinants. So the use of erythromycin can also increase the rate of clindamycin resistance among *B. fragilis* (7).

Considering the fact that 99% of fecal isolated *B. fragilis* species produce beta-lactamase (7), cephalosporins such as cefotaxime and cefoxitin have gradually lost their effects. For example resistance against cefoxitin has been reported from different countries (6, 14, 16) and the highest resistance level against this antibiotic among *B. fragilis* is in Taiwan (19). This issue has been reported in the results of this research and also in findings of others (6, 14, 16, 19). The importance of this issue could be understood better when it is considered that these beta-lactam antibiotics even along with beta-lactamase enzyme inhibitors such as amoxicillin in addition to clavulanic acid, also has lost a high percentage of their effectiveness against *Bacteroides* (12). This kind of resistance against beta-lactam antibiotic plus beta-lactamase inhibitor shows development of another method of resistance. On the other hand, the results obtained in this research showed a good effect of piperacillin plus tazobactam on isolated *B. fragilis* which is completely in accordance with reports of other researchers (12, 14).

Using chloramphenicol in most countries has been reduced due to high toxicity of drug including leading to intestinal disorders and anemia. But high consumption of this antibiotic in some countries leads to observation of high resistance *B. fragilis* against this antibacterial material (5). This resistance may be due to transfer of resistance determinants or irregular consumption of antibiotic. Two different types of chloramphenicol resistance have been detected in *B. fragilis* where both resist by drug deactivation through nitro reduction in p-nitro group in benzene circle or through acetylation (1). According to available information in areas where they are still using this drug against infections caused by *Bacteroides* or other bacteria, there is a resistance against chloramphenicol (5). The results of this research also show some resistance (55%) against this antibiotic, among isolated *Bacteroides*.

Rifampin resistance is not seen among *B. fragilis* strains isolated from clinical samples or even from human natural flora, so resistance to rifampin 15µg disk is considered as one of identifying tests for *Bacteroides* (Table 1). But experiences have shown that rifampin resistance *B. fragilis* has been rarely observed after therapy of patients' urethral tracts infections and tuberculosis using this antibiotic (20). Historically, fluoroquinolones are not considered as good and effective anti-microbial materials against anaerobic bacteria. Resistance mechanism against this antibiotic is very similar to facultative aerobics and is achieved by mutating in gyrA related gene or by increased expression of efflux pump. The results of this research show 100% resistance against ciprofloxacin which is in complete accordance with reported results of Garcia et al (21).

According to obtained results, *B. fragilis* are still sensitive to important antibiotics that are effective on anaero-

bic bacteria, such as imipenem, metronidazole, piperacillin/tazobactam and on the other hand, they gained high resistance against some other antibiotics that previously were used in anaerobic infections.

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Authors' Contribution

None declared.

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