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Research Paper

Prevalence and antibiotic susceptibility of *Bacteroides fragilis* group isolated from stool samples in North Lebanon

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Abstract

Fifty one strains of the *Bacteroides fragilis* group were isolated from 45 fecal samples. Classical phenotypic identification showed that 16 isolates were *B. thetaiotaomicron*, 12 *B. uniformis*, 9 *B. eggerthii*, 7 *B. vulgatus*, 3 *B. caccae*, 2 *Parabacteroides distasonis* with 1 identified *B. ovatus* and 1 *B. fragilis*. The 51 strains were tested for susceptibility against 16 antimicrobial agents and the MICs for metronidazole were determined. The tests showed that imipenem, meropenem and chloramphenicol were the most effective antibiotics (98%, 98% and 92.16% of susceptibility, respectively) followed by ticarcillin/clavulanic acid, piperacillin/tazobactam, rifampin (88.24% susceptibility), moxifloxacin 86.27% and tigecycline 84.31%. Ofloxacin and cefotaxime were the least effective antibiotics with 27.45% and 0% of activity respectively. Only six of the 51 isolated strains were resistant to metronidazole with MICs = 64 mg/L (1 strain) and > 256 mg/L (5 strains).

Key words: *Bacteroides fragilis* group, anaerobic bacteria, antibiotics susceptibility.

Introduction

Bacteroides fragilis group are gram-negative, anaerobic, non spore-forming and bile resistant bacteria. They are part of the endogenous microbiota of humans and other mammals (Aldridge and O'Brien, 2002; Rodriguez *et al.*, 2006). The species most frequently isolated from the flora are *B. thetaiotaomicron*, *B. vulgatus* and *P. distasonis* and to a less extent *B. eggerthii* and *B. fragilis* (Holdeman *et al.*, 1976).

Members of this group have the potential to be opportunistic pathogens when they are transferred to a normally sterile site (Smith *et al.*, 2006). Moreover, they are clinically important and constitute more than a third of anaerobes isolated from clinical samples (Goldstein, 1996).

The *Bacteroides* are frequently isolated from mixed infections involving aerobic and anaerobic bacteria such as intra-abdominal infections (Aldridge, 1995). They are also responsible for gynecological infections (Bergan, 1983), endocarditis and pericarditis (Brook, 2002a), bacteremia (Brook, 2002b), *B. fragilis* - one of the 10 species of the *B. fragilis* group - is the most frequently isolated from blood cultures (Aldridge, 1995).

The choices of antibiotics for treatment are limited because the species of the *Bacteroides fragilis* group are among the most resistant anaerobic to antimicrobial agents (Syndman *et al.*, 2002).

The most frequently prescribed antibiotics include β-lactams, carbapenems, clindamycin, metronidazole. Fluoroquinolons are also prescribed in combination with either clindamycin or metronidazole (Brook, 2002c).

Resistance to the most active drugs, such as imipenem, piperacillin-tazobactam, ampicillin-sulbactam, and metronidazole, is reported in occasional strains (Pumbwe *et al.*, 2007).

The antimicrobial susceptibility varies between species of this group (Aldridge and O'Brien, 2002) and *B.fragilis* being more susceptible to numerous antimicrobial agents than other species of the *B. fragilis* group (Wexler, 2007).

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In Lebanon, we lack national data about the prevalence of various species of *Bacteroides fragilis* group in the intestinal flora and their susceptibility to antibiotics.

The aim of this work was to determine the prevalence of *Bacteroides fragilis* group species in 45 diarrheal and non-diarrheal stools samples, their antibiotic susceptibility and finally to determine the minimal inhibitory concentration (MIC) to metronidazole by using E-test.

Materials and Methods

Location and period of the study

The study took place in the microbiology laboratory at the AZM center for biotechnology research and its applications - Lebanese University - in Tripoli, northern Lebanon, between May 30 and July 25, 2011.

Sampling and transporting of samples

The study was made feasible through collaboration with several hospitals laboratories: Nini, Haykal, Islami, Mazloum and Monla.

Upon receipt of the stool, the technician in charge swabbed the stool. To preserve the viability of *Bacteroides*, the transport system Port-A-Cul (Beckton-Dikinson, USA) was used. This system contains a medium suitable for the transport of anaerobic bacteria.

Promptly after collection, the samples were transported to the center for analysis.

45 stool samples with different characteristics (liquid, viscous and solid) were collected.

Isolation of *Bacteroides*

Upon receipt, the contents of each swab was spread on the surface of a regenerated Columbia base agar (Biorad, France) supplemented with 5% blood, hemin 0.005 g/L (Sigma, Germany) and vitamin K 0.01 g/L (Sigma, Germany).

This medium is made selective by the use of the following antibacterial agents: Amikacin (0.01 g/L) and vancomycin (0.004 mg/L) and antifungal agent: mycostatin (240 U/L).

Immediately after inoculation, the plates were rapidly incubated in anaerobic atmosphere, using an Oxoid type jar and GENbox bags (Biomérieux, France). The anaerobic environment was confirmed via the introduction of an anaerobiosis indicator - Anaer indicator strips (Biomérieux, France).

After closing, the jar was incubated at 35-36 °C for 48 hours.

To control the medium used and the culture conditions, the following reference strains were used: *B. fragilis* ATCC 25285, *B. uniformis* ATCC 8492, *B. vulgatus* ATCC 29327, *B. ovatus* ATCC 8483.

Characterization of isolates

The colonies having these following aspects, smooth - white to gray - non hemolytic and 1 to 3 mm in diameter, were gram stained and the respiratory type was determined by using the standard tube method according to Marchal *et al.* (1982) which is based on the culture of the suspected colonies in a regenerated meat-liver medium (Biorad, France) placed in a tube of 9 x 180 mm of size, as result the anaerobic bacteria have grown at the bottom of the agar. A subculture was performed for each colony that showed the presence of anaerobic gram negative bacilli.

The full identification was obtained using a biochemical gallery RapID^{TM}ANAII (Remel Company, USA) whose protocol was followed. The inoculum turbidity was equal to 3 McFerland turbidity or equivalent and the incubation was performed in a non-CO2 incubator for 4 to 6 hours at 37 °C.

The same reference strains were used for the quality control.

Susceptibility of isolates to antibiotics

The susceptibility test was realized by the disk diffusion method and according to the protocol proposed by the committee of antibiogram of the French Microbiology Society (CA-SFM, 2011). The bacterial suspension was prepared in regenerated NaCl 5% and had a turbidity equivalent to 1 McFarland (10^8 UFC/mL). This suspension was inoculated onto Wilkins-Chalgren agar medium (Beckton-Dikinson, USA) supplemented with 5% of horse blood.

The interpretation of results was done after 48 hours of incubation at 37 °C in an anaerobic atmosphere.

We used the discs commercialized by Biorad®-France as followed: Amoxicillin + Clavulanic Acid AMC (20/10 µg), Ticarcillin + Clavulanic Acid TCC (75/10 µg), Piperacillin + Tazobactam TZP (100/10 µg), Cefoxitin FOX (30 µg), Cefotaxime CTX (30 µg), Imipenem IPM (10 µg),Meropenem MEM (10 µg), Kanamycin K (30 µg), Vancomycin VA (30 µg), Colistin CS (10 µg), Chloramphenicol C (30 µg), Rifampin RA (30 µg), Ofloxacin OFX (5 µg), Moxifloxacin MXF (5 µg), Clindamycin CM (2 µg), Tigecyclin TGC (15 µg).

Finally, we determined the MIC of the isolates to metronidazole using the E-test method. We used the strips commercialized by Biodisk-Solna, Sweden, distributed in Lebanon by Biomérieux-France. The test was performed according to the protocol suggested by the CA-SFM (2011).

Results

In total, we analyzed 45 stools and after isolation, 51 *Bacteroides* strains identified by the RapID^{TM}ANAII device, were investigated (Table 1).

Table 1 - Distribution of *Bacteroides fragilis* group species according to age and specimen condition.

Age	Aspect	*B. thetaiotaomicron*	*B. uniformis*	*B. eggerthii*	*B. vulgatus*	*B. caccae*	*B. distasonis*	*B. fragilis*	*B. ovatus*	Total
< 18 ans	D	6 (11.76)	4 (7.85)	6 (11.76)	2 (3.92)	3 (5.88)			1 (1.96)	22 (43.13)
	N.D	5 (9.81)	1 (1.96)		3 (5.88)		1 (1.96)			10 (19.61)
19-54 ans	D	1 (1.96)	1 (1.96)				1 (1.96)			3 (5.88)
	N.D	4 (7.85)	4 (7.85)	3 (5.88)	2 (3.92)					13 (25.50)
> 54 ans	D		2 (3.92)					1 (1.96)		3 (5.88)
	N.D									
Total		16 (31.37)	12 (23.53)	9 (17.65)	7 (13.73)	3 (5.88)	2 (3.92)	1 (1.96)	1 (1.96)	51 (100)

D = diarrheal N.D = non diarrheal (%) = percentage of strains.

Note: there were 6 samples from which we isolated two different species of the *Bacteroides fragilis* group.

Results of the antimicrobial susceptibility of the *Bacteroides fragilis* group strains, are summarized in Table 2.

The results of MICs of metronidazole determined by the E-test method were: 45 strains with an MIC ≤ 4 µg/mL, 1 strain with an MIC = 64 µg/mL and 5 strains with an MIC > 256 µg/mL of which 3 were *B. thetaiotaomicron*, 1 *B. vulgatus* and 1 *B. distasonis*.

Discussion

In this study we looked for *Bacteroides fragilis* group in 45 stools of which 24 (53.33%) were diarrheal and 21 (46.67%) non-diarrheal specimens. After culture and biochemical identification 51 isolates were identified amongst the *Bacteroides fragilis* group.

Biochemical identification revealed that *B. thetaiotaomicron* was the predominant species (31.37%) followed by *B. uniformis* (23.53%), *B. eggerthii* (17.65%), *B. vulgatus* (13.73%), *B. caccae* (5.88%), *P. distasonis* (3.92%), and *B. ovatus* and *B. fragilis* (1.96%), respectively.

In a study performed by Ferreira *et al.* (2010) in Brazil, the authors reported entirely different results, where *P. distasonis* was the major species (19.1%), followed by *B. fragilis* (17.0%), *B. vulgatus* (17.0%), *B. caccae* (2.1%) and *P. merdae* (2.1%).

In the case of diarrheal stools *B. thetaiotaomicron and B. uniformis* were the dominant species (13.73%, respectively), followed by *B. eggerthii* (11.76%).

We also found that *B. thetaiotaomicron* and *B. eggerthii* (27.27%, respectively) were the major species in diarrheal stools of children under 18 years old followed by *B. uniformis* (18.18%), *B. caccae* (13.64%), *B. vulgatus* (9.09%) and *B. ovatus* (4.54%) (Table 1).

Nakano and Avila-Campos (2004) reported in a study performed on stools of 15 children with diarrhea the isolation of 64 *B. fragilis* group strains of which 39 were *B. fragilis*, 8 *B. vulgatus*, 6 *B. uniformis*, 6 *P. distasonis* and 5 *B. ovatus*. In our study we isolated a single strain of *B. fragilis* in diarrheal stool of a person older than 54 years (74 years).

As for the non-diarrheal stools, we found that *B. thetaiotaomicron* was the most frequent isolate (17.65%) followed by *B. uniformis* and *B. vulgatus* (9.80%, respectively), *B. eggerthii* (5.88%) and *P. distasonis* (1.96%).

Rodriguez *et al.* (2006) studied the prevalence of different species of the *B. fragilis* group; they analyzed 80 non-diarrheal stools and according to their results, *B. uniformis* and *B. fragilis* were the most frequent species (24.6% and 16.9%, respectively).

Antibiotic resistance

We studied the susceptibility of 51 isolates belonging to the *Bacteroides fragilis* group. We found that 60.78% and 88.24% of the investigated strains were susceptible to clavulanic acid combined with either amoxicillin or ticarcillin. Tazobactam combined with piperacillin provided results similar to those of ticarcillin plus clavulanic acid (88.24%).

Therefore, the non suscptibility rates for these three drugs were 39.21%, 11.76% and 11.76%, respectively (Table 2).

A higher rate of resistance to amoxicillin-clavulanic acid (40.5%) was observed in a study performed by Nakano and Avila-campos (2004) on 64 *Bacteroides fragilis* group strains isolated from children with diarrhea in Brazil.

Only 4% of resistance was detected in a study performed by Oteo *et al.* (2000) on 100 isolates of *Bacteroides fragilis* group isolated from fecal samples of healthy people in Madrid, Spain.

In our study, the highest activity was observed with imipenem and meropenem where 98% of strains were sensitive to these antibiotics molecules (we found that one of seven *B. vulgatus* strains isolated was resistant to imipenem and meropenem) (Table 2).

Nakano and Avila-Campos (2004) showed in their study that none of 64 *Bacteroides fragilis* group strains isolated from human feces were resistant to imipenem.

A very good activity of meropenem has been reported by Oteo *et al.* (2000) With 100% of susceptibility, but a higher rate of resistance was reported by Ferreira *et al.* (2010) (18.2%).

Concerning the cephalosporins, we found that 68.63% of the strains were susceptible to cefoxitin (non susceptibility rate = 31.37%) while 13.73% had intermediate susceptibility to cefotaxime (86.27% resistance) (Table 2).

Different rates of cefoxitin resistance have been reported in several studies performed by Nakano and Avila-Campos (2004) in Brazil (23.5%) and Oteo *et al.* (2000) in Spain (18%).

For quinolones, susceptibility to ofloxacine was 27.45% while moxifloxacin susceptibility was 86.27%.

The susceptibility to clindamycin was 54.9% (resistance = 45.1%) (Table 2).

Oteo *et al.* (2000) Ferreira *et al.* (2010) and Nakano and Avila-Campos (2004) reported high values of resistance (49%, 22.7% and 23.5%, respectively) in studies performed in Spain and Brazil.

In this study, the antibiotic susceptibilty to other molecules were: 92.16% to chloramphenicol (resistance =

Table 2 - Results of the antimicrobial susceptibility of the *Bacteroides fragilis* group strains.

Antibiotic	Clinical categorization of the clinical strains according to breakpoints in %					
	Susceptible*		Intermediate		Resistant	
	N°	%	N°	%	N°	%
AMC	31	60.78	2	3.92	18	35.29
TCC	45	88.24	1	1.96	5	9.80
TZP	45	88.24			6	11.76
IPM	50	98			1	2
MEM	50	98			1	2
FOX	35	68.63	9	17.65	7	13.72
CTX			7	13.73	44	86.27
OFX	14	27.45	7	13.7	30	58.82
C	47	92.16			4	7.84
RA	45	82.24	1	1.96	5	9.80
CS	5	9.80			46	90.20
K	2	3.92			49	96.08
V	4	7.84	7	13.73	40	78.43
TGC	43	84.31			8	15.69
MXF	44	86.27			7	13.73
CM	28	54.9			23	45.1

AMC = amoxicillin/clavulanate TCC = Ticarcillin/clavulanate TZP = piperacillin/tazobactam IPM = imipenem MEM = meropenem FOX = cefoxitin CTX = cefotaxime OFX = ofloxacin C = chloramphenicol RA = Rifampin CS = colistin K = kanamycin VA = vancomycin TGC = tigecycline MXF = moxifloxacin CM = clindamycin. N° = number of strains.
*According to the recommandation of CA-SFM (2011).

7.84%) 82.24% to rifampicin (non susceptibility = 11.76%) and 84.31% to tigecycline (resistance = 15.69%).

Finally, we found a false susceptibility to kanamycin, colistin and vancomycin (3.92%, 9.80% and 7.84%, respectively) (Table 2), false sensitivity was detected because these three antibiotics are disks with special potency that helps to presumptive identification of anaerobes, they are not done to determine antibiotic susceptibility.

However some rare strains of the *Bacteroides fragilis* may have MIC of 16 mg/L and may appear susceptible to vancomycin.

In addition we didn't use disks with special potency aimed for the identification of anaerobes, instead we used regular disks.

The highest non susceptibility rate to cefoxitin was observed in *B. thetaiotaomicron* (37.5%).

The highest non susceptibility rates to clindamycin, ofloxacin and moxifloxacin was observed in *B. uniformis* (58.33%, 75% and 91.67%, respectively).

The best susceptibility to moxifloxacin was observed for *B.vulgatus* (100%), followed by *B. eggerthii* (88.99%) and *B. thetaiotaomicron* (81.25%).

As for chloramphenicol, 100% of the isolates belonging to *B. uniformis* were susceptible, followed by *B. eggerthii* (88.89%) and *B. thetaiotaomicron* (87.5%).

100% of *B. vulgatus* isolates studied were susceptible to tigecycline followed by *B. eggerthii* (88.89%) and *B. thetaiotaomicron* and *B. uniformis* (75%, respectively) (Table 3).

Avelar *et al.* (2001) showed that *P. distasonis* was the most resistant species to cefoxitin and clindamycin in a study performed in Brazil on 73 strains of *Bacteroides fragilis* group.

Susceptibility to metronidazole

We determined the MIC of metronidazole for the 51 isolates by the E-test method.

We found that there were 6 resistant strains (11.76%), a strain belonging to the species *thetaiotaomicron* had a MIC = 64 mg/L and five strains had MIC values > 256 mg/L.

Note that a strain of *B. vulgatus* had a MIC = 0.016 mg/L, four strains of *B.thetaitaomicron* had MIC = 0.064 and 0.125 mg/L, respectively.

The rate of resistance is significantly higher compared to values reported in studies of strains isolated in Brazil and Spain (0%, respectively) (Ferreira *et al.*, 2010; Nakano and Avila-Campos, 2004; Oteo *et al.*, 2000).

Conclusion

Our study is original in nature as it is the first in Lebanon on the prevalence and distribution of the *Bacteroides fragilis* group species in diarrheal and non-diarrheal stools in North Lebanon.

Disk diffusion test may not be for some authors the best reference method, nevertheless this sudy allow document the general susceptibility of the B fragilis group strains in Lebanon.

The results showed that *B. thetaiotaomicron* was the most prevalent species in diarrheal and non-diarrheal stools.

We observed a high rate of resistance to cefoxitin (31.37%).

Imipenem and meropenem showed excellent activity (98% of susceptibility) meanwhile13.73% of our isolates were resistant to moxifloxacin. An alarming rate of resistance to clindamycin was observed (45.1%).

Table 3 - Susceptibility percentage of the four major species of the *Bacteroides fragilis* group isolated to tested antibiotics.

	B. thetaiotaomicron	*B. uniformis*	*B. eggerthii*	*B. uniformis*
	Antibiotic susceptibility %			
AMC	50	75	55.56	71.43
TCC	75	100	100	100
TZP	81.25	91.67	100	100
IMP	100	100	100	85.71
MEM	100	100	100	85.71
FOX	62.5	66.66	66.67	71.42
CTX	0	0	11.11	0
OFX	18.75	8.33	55.56	42.86
C	87.5	100	88.89	85.71
RA	68.75	100	100	85.71
TGC	75	75	88.89	100
MXF	81.25	75	88.89	100
CM	43.75	41.67	77.78	71.43

After the determination of MIC of metronidazole, the results showed that 11.76% of our strains were resistant.

It is advisable to continue this investigation, in attempt to identify genes that are involved in different resistance mechanisms observed. This study emphasizes the need for antibiotic surveys of anaerobes in the future.

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