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Original Article

Molecular characterization of Multidrug Resistant *Bacteroides* isolates from Hungarian clinical samples

Running title:

Investigation of MDR *Bacteroides* isolates from Hungarian clinical samples

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Highlights

- In a multicentre study, the antibiotic susceptibility of 400 Bacteroides isolates were tested for 10 antibiotics.
- Six MDR strains were found and their antibiotic resistance genes were investigated by molecular methods.
- In this study we demonstrated the relatively large number of MDR strains within a comprehensive multicenter survey from one clinical centre.
- We emphasized the importance of antimicrobial susceptibility testing and surveillance among *B. fragilis* group isolates.

Abstract

Introduction: Members of the *Bacteroides fragilis* group are the most important components of the normal human gut microbiota, but these bacteria can cause severe infections as well. Due to the frequent usage of antibiotics, the spreading of the MDR strains is a real threat worldwide.

Materials and methods: In a multicentre study 400 *Bacteroides* isolates from five Hungarian microbiological laboratories were cultured and identified by MALDI TOF-MS. The MIC values of ten antibiotics were determined by the agar dilution method and evaluated by the breakpoints of EUCAST or CLSI.

Results: We found six MDR strains, and their antibiotic resistance genes were investigated by molecular methods.The DNA amplicon of *B. fragilis* SZ38 strain was sequenced to look for a mutation in the *gyrA* gene. Among the six MDR isolates we found one *cfiA*-, two *cepA*, three *cfxA*-, two *ermG*-, six *tetQ*- three *tetX*- and two *bexA*-positive strains. None of them harboured *cepA, nim, ermB* and *tetX1* genes.

Discussion: In the past 12 years only a few cases of MDR *Bacteroides* infections have been published. Within a comprehensive multicenter survey we demonstrated the relatively large prevalence of MDR strains isolated in one centre with five isolates and one from another centre during a relatively short period of time. This study focused on the importance of antimicrobial susceptibility testing and surveillance among *B. fragilis* group isolates.

Keywords: MDR *Bacteroides* isolates, antibiotic resistance genes, agar dilution, sequencing, RT-PCR

1 Introduction

Although the *Bacteroides* species are members of the normal human gut microbiota, these bacteria are significant opportunistic pathogens as well and are responsible for significant mortality, especially in the case of bacteremia [1,2]. *B. fragilis* can cause intraabdominal, skin and soft tissue infections and diarrhoea caused by enterotoxin producing *B. fragilis* isolates, particularly in young children under the age of five years [1,3]. The most important antibiotics against anaerobes are cephamycins, β-lactam/ β-lactamase inhibitors, carbapenems, metronidazole, clindamycin and fourth generation fluoroquinolones [1]. Nagy

et al. reported a high rate of cefoxitin, clindamycin and moxifloxacin resistant strains, but amoxicillin/clavulanic acid, piperacillin/tazobactam, carbapenems and metronidazole are still very active antibiotics against *B. fragilis* group strains [4]. Antibiotic resistance is mediated by chromosomal genes or extrachromosomal plasmids, transferred by different types of transposons; and some genes require insertion sequence (IS) elements upstream of the gene for expression. Resistance to β-lactams is mediated mostly by chromosomal β-lactamase, encoded by *cepA* or *cfxA* genes, while carbapenem resistance is associated with *cfiA* gene by the expression of metallo-*β*-lactamase [5]. Although the mechanism of metronidazole resistance is not yet fully elucidated, the *nim* genes (*nimA-H, nimJ*) are thought to be responsible for it [6]. Ribosomal protection protein encoded by *tetQ* gene and enzymatic modification of the tetracycline molecule (*tetX, tetX1*) can cause tetracycline resistance [5,7]. Also, Macrolide-Lincosamide-Streptogramin B resistant determinants, *erm(A-F, H)* are widely distributed among *Bacteroides* species [5]. Mutations of the Quinolone Resistance-Determining Region (QRDR) of the genes of gyrase (*gyrA, gyrB*) and/or topoisomerase IV (*parC, parE*), besides increased efflux, as in the Multi-Antimicrobial Extrusion protein (MATE-family, encoded by the *bexA* gene) may develope fluoroquinolone resistance [5,8]. In a recent Hungarian antibiotic resistance survey we tested six MDR *Bacteroides* isolates.

2 Methods

2.1. Bacterial strains and cultivation

In our study, 400 *Bacteroides* isolates collected from clinically relevant samples by five Hungarian clinical microbiological centres between January 2014 and March 2016 were investigated. The strains were stored at -80 °C in Brain Heart Infusion (BHI) broth with 20% glycerol and cultured on Schaedler agar (bioMérieux, Fr.) for 48 hours, at 37 °C in an anaerobic chamber (Perkin Elmer, UK) under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2). The strains were identified with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker Biotyper, Germany) and a Biotyper Version 3.0 software package was used and the isolates yielded a high confidence species level identification. The *B. fragilis* strains were identified as genetic Division II (exclusively positive for the *cfiA* carbapenemase gene) and Division I by the MALDI-TOF MS identification scheme published previously [9].

2.2. Agardilution method

The Minimal Inhibitory Concentration (MIC) values of 10 antibiotics (ampicillin, amoxicillin/clavulanic acid, cefoxitin, meropenem, clindamycin, moxifloxacin, metronidazole, tetracycline, tigecycline, chloramphenicol) of all the strains were determined with the agar dilution method according to the recommendation of Clinical and Laboratory Standard Institute (CLSI) [10]. Steers-Foltz's replicator was used to place 10^5 CFU per spot onthe surface of Brucella agar supplemented with 5% laked sheep blood containing the antibiotics [10]. The following concentrations of the antibiotics were tested: ampicillin (2-256 mg/l), amoxicillin/calvulanic acid (0.064-16 mg/l), cefoxitin (0.5-256 mg/l), meropenem (0.064-16 mg/l), clindamycin (0.064-256 mg/l), metronidazole (0.064-8 mg/l), moxifloxacin (0.064-32 mg/l), tetracycline (0.125-256 mg/l), tigecycline (0.064-32 mg/l), chloramphenicol (0.125-32 mg/l). We used fixed concentration of amoxicillin/clavulanic acid for stock solution (10/2.5 mg/ml). After 48 h incubation at 37 °C in an anaerobic chamber (Perkin Elmer, UK) under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2), the ampicillin, amoxcillin/clavulanic acid, meropenem, clindamycin, metronidazole MIC values were evaluated based on breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST); however, cefoxitin, tetracycline, moxifloxacin and chloramphenicol were evaluated by the breakpoints of CLSI (Table 1) [10,11]. As neither EUCAST nor CLSI provided MIC values of tigecycline, we interpreted these MICs according to the data of the study published by Nagy *et al*. [4]. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were applied as control strains. Among the 400 strains, we found six MDR isolates. The clinical data of patients, who were infected by MDR *Bacteroides* isolates and their MIC values, are summarized in Table **2.3. RT-PCR**

Molecular investigations of the MDR isolates were carried out in order to detect most common antibiotic resistance genes (*cepA, cfxA, cfiA, nim, ermB, ermF, ermG, tetQ, tetX, tetX1, bexA),* IS*4351*, the upstream region of *cfiA* and *cfxA* genes. In the case of *gyrA, gyrB, parC* and *parE* genes we looked for amino acid substitutions. DNA templates then were prepared by using the colony boiling lysis method. RT-PCR reactions were performed to detect *cepA, cfxA, cfiA, ermF, ermB, ermG, tetQ, tetX, tetX1, bexA, gyrA* genes and IS*4351*;

while the end-point PCR method were used for the amplification of the upstream region of *cfiA, cfxA genes* and IS*4351*. The PCR products were analysed with 1.2% agarose gel electrophoresis; and the whole PCR set-up is summarised in Table 2. Positive controls used were the following: *B. fragilis 638R* (*cepA*), *B. vulgatus* CLA341 (*cfxA, tetQ*), *B. fragilis* 638R (*nim*), *B. fragilis* (*ermF*), *B. thetaiotaomicron* (*ermG*), *B. fragilis* BM13 (*tetX1*), *B. fragilis* pBRT21 (*bexA*) [12].

2.4. Sequencing of the *gyrA* gene

The DNA amplicon of the SZ38 *B. fragilis* strain (proportional scale-up to 30 µl) was purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan). And the PCR products were sequenced with ABI BigDye® Terminator Version 3.1 kit in the Series Genome Analyser 3500 (Life Technologies).

3 Results

A multicentre national survey was performed with 400 *Bacteroides* strains and six MDR isolates were found: one *B. fragilis*, two *B. ovatus*, two *B. vulgatus* and one *B. thetaiotaomicron*. As for the geographical distribution, one MDR strain was isolated from Debrecen (*B. ovatus* D92) and five from Szeged (*B. vulgatus* SZ4, SZ34 *B. ovatus* SZ9, *B. thetaiotaomicron* SZ35 and *B. fragilis* SZ38) were found, but none from the other centres (Table 1). Most of the MDR isolates in question displayed resistance to ampicillin (n=6), cefoxitin (n=4), moxifloxacin (n=5), clindamycin (n=4) and tetracycline (n=5), with a range of resistance from four to six different antibiotic classes. The results of the genetic analysis are summarised in Table 3. The *B. fragilis* SZ38 isolate harboured the *cfiA* gene with a high level resistance to ampicillin, amixicillin/calvulanic acid, cefoxitin, meropenem, but without any IS-element in the upstream region. None of the strains harboured the *cepA* gene, and three *cfxA* positive isolates (*B. vulgatus* SZ4, *B. ovatus* SZ9 and *B. thetaiotaomicron* SZ35) were detected. The 1.2 kb regulator region of the *cfxA* gene of the *B. vulgatus* SZ4 isolate was found. Four strains (*B. ovatus* D92, SZ9, *B. vulgatus* SZ34, and *B. thetaiotamicron* SZ35) expressed a high level of clindamycin resistance (MIC>256 mg/l), *B. ovatus* D92 harboured the*ermG* gene, while *B. vulgatus* SZ4, *B. thetaiotaomicron* SZ35 *ermF* gene,and *B. ovatus* SZ9 contained both of them. The full length of IS*4351* was detected in *B. vulgatus* SZ4 and

B. thetaiotaomicron SZ35 strains, but we did not observe any physical association of these IS-s with *ermF* genes using PCR mapping. All of the isolates harboured the *tetQ* gene and three of them (*B. ovatus* D92, SZ9 and *B. fragilis* SZ38) expressed a high level tetracycline resistance (MIC>32 mg/l); moreover, the *B. ovatus* SZ9, *B. vulgatus* SZ34 and *B. thetaiotaomicron* SZ35 strains contained the *tetX* gene simultaneously. None of the isolates harboured the *nim* gene, but the *B. ovatus* D92 strain was metronidazole resistant based on the EUCAST breakpoints. The fluoroquinolone susceptibility test was performed with the measurement of moxifloxacin MIC-values. In the case of four strains, moxifloxacin MICs?32 mg/l were detected, and among them the *B. thetaiotamicron* SZ35 harboured the *bexA* gene. Point mutations were investigated in the case of the *gyrA* gene of the *B. fragilis* SZ38 strain, and with a sequence analysis Ser82?Phe substitution in the QRDR region of the GyrA subunit of gyrase enzyme was detected. Tigecycline and chloramphenicol are very active against these isolates, both of them being susceptible to these two drugs.

4 Discussion

To date, MDR *Bacteroides* isolates have been rarely published. In the past decade, cases involving three Americans [13,14], one Briton [15], two Greeks [16], and one Japanese [17] have been published in the literature and one article published an investigation of five Danish isolates [6]. The case of an American soldier was also published, who suffered serious injuries in Afghanistan [18]. In our comprehensive, multicentre study we found six MDR isolates of 400 *Bacteroides* strains, which showed resistance to four to six different antibiotic groups. The molecular background of the resistance pattern of the MDR isolates differ from strain to strain. In Hungary, only one MDR *B. fragilis* isolate has been published so far, which was resistant to penicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, meropenem, clindamycin and tetracycline, harboured *cepA, cfiA, erm, nimA, tetQ* genes and IS*1187* element [19]. The antibiotic resistance pattern, as well as the molecular background of the MDR *Bacteroides* isolates differ based on geographic location, type of isolate, species and antibiotic usage [19]. In the upstream region of the *cfxA* gene, special genetic elements (IS*614B*, Tn*4555* and Tn*4351*) can be usually detected, (which were described earlier by Garcia *et al.* and Sóki *et al.* [20,21], but *B. ovatus* SZ9 and *B. thetaiotamicron* SZ35 harboured another IS-element or deletion. A Danish study reported five MDR *B. fragilis* blood culture isolates that harboured *cfiA, nimA, nimD, nimE, nimJ,*

tetQ, *ermB*, *ermF*, *LinA2* (clindamycin resistance), *cepA*, *cfxA*, *bexB* genes and different IS elements (IS*614B*, IS*613B*, IS*1169*, IS*1187*, IS*1188*, IS*4351*, IS*Bf6*, IS*Bf12*, IS*Bf13*) [6]. *B. fragilis* SZ38 strain harboured *cfiA* gene, without any IS-element in the upstream region. None of our strains harboured the *cepA* gene, and three *cfxA* positive isolates were detected. Four strains harboured the *ermG*, *ermF* gene or both of them. The full length of IS*4351* was detected in *B. vulgatus* SZ4 and *B. thetaiotaomicron* SZ35 strains. All of the isolates harboured the *tetQ* gene; moreover, the three strains contained the *tetX* gene simultaneously. Two strains harboured *bexA* gene; and none of them the *nim* gene. Ser82Phe substitution was found in GyrA region of the *B. fragilis* SZ38 strain, as well as Nakamura *et al.* reported in the case of MDR *B. fragilis* isolate [17].

5 Conclusions

The novelty of this study is that we demonstrated a relatively significant prevalence of MDR strains isolated in Szeged with five isolates and another strain from Debrecen. In the Central Eastern European region, up till now no similar study has reported such a large number of MDR *Bacteroides* strains. Almost all of them were resistant to ampicillin, cefoxitin, clindamycin, moxifloxacin and tetracycline, and harboured resistance genes and/or genetic elements related to the antibiotic resistance, according to the detailed molecular investigation. The background of the increasing number of MDR strains in the Szeged region may be the local habit of antibiotic usage, which might have led to an elevated level of resistance to several different antibiotics. This study demonstrates the importance of antimicrobial susceptibility testing and surveillance among *B. fragilis* group isolates. The increasing antibiotic resistance may be responsible for therapeutic failure and the higher mortality rate.

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Competing interest: None.

Ethical approval: Not required.

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Table 1 Data of the MDR *Bacteroides species* strains

Isolate	Pure or mixed culture	Age & gender	Clinical presentation	Sample type	Department of isolation	MIC values (mg/l)										Treatment	Outcome
						AMP (>2[a])	AUG (>8[a])	FOX (≥64[b])	MER (>8[a])	CLI (>4[a])	MTZ (>4[a])	MOX (≥8)	TET (≥16[b])	TIG ND	CHL (≥32[b])		
D92 ***B. ovatus***	m	68 M	Diabetes mellitus, atherosclerosis, renal failure, leg amputation	Decubitus	Internal medicine	>256	>16	32	1	>256	>8	32	32	4	8	Meropenem, clindamycin, vancomycin, colistin	Alive
SZ4 ***B. vulgatus***	m	64 M	Diabetic foot	Wound	Surgery	>256	>16	128	4	1	0,25	>32	16	0,25	4	Ciprofloxacin Metronidazole	Deceased
SZ9 ***B. ovatus***	m	73 F	Appendicitis	Intraabdo-minal fluid	Surgery	>256	16	32	16	>256	0,5	32	32	0,5	8	Cefuroxime+Metronidazole Ciprofolxacin+ Metronidazole	Alive
SZ34 ***B. vulgatus***	m	63 M	*C. difficile* colitis	Autopsy	Pathology	>256	>16	128	4	>256	0,5	32	8	0,125	4	ND	Deceased
SZ35 ***B. thetiotaomicron***	m	67 F	Abrasion	Intrauterine device	Obstetrics & Gynecology	>256	16	128	2	>256	0,5	1	16	0,25	8	No antibiotic treatment	Alive
SZ38 ***B. fragilis***	m	76 M	Diabetic foot, atherosclerosis, leg amputuation	Wound	Surgery	>256	>16	128	16	8	0,5	8	32	0,5	4	Meropenem	Deceased

AMP: Ampicillin, AMC: Amoxicillin/clavulanic acid, FOX: Cefoxitin, MER: Meropenem, CLI: Clindamycin, MZT: Metronidazole, MOX: Moxifloxacin, TET: Tetracycline, TIG: Tigecycline, CHO: Chloramphenicol

m: mixed culture M: male F: female [a] EUCAST breakpoints [b] CLSI breakpoints ND: No data

Table 2 PCR reaction parameters of the genes and genetic elements examined

Gene	Primers (5' → 3')	PCR cycles
cfiA	AATCGAAGGATGGGGTATGG	95 °C 15 s, 59 °C 30 s, 72 °C 30s, 35x
	CGGTCAGTGAATCGGTGAAT	
cfxA	TGACTGGCCCTGAATAATCT	95 °C 15 s, 55 °C 30 s, 72 °C 30s, 35x
	ACAAAAGATAGCGCAAATCC	
cepA	TTTCTGCTATGTCCTGCCT	95 °C 15 s, 56 °C 30 s, 72 °C 1 min, 35x
	ATCTTTCACGAAGACGGC	
nim	ATGTTCAGAGAAATGCGGCGTAAGTG	94 °C 15 s, 62 °C 30 s, 72 °C 30 s, 35x
	GCTTCCTCGCCTGTCACGTGCTC	
ermF	TAGATATTGGGGCAGGCAAG	95 °C 15 s, 58 °C 1 min, 72 °C 30 s, 35x
	GGAAATTGCGGAACTGCAAA	
ermB	GCGGAATGCTTTCATCCTAA	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35x
	GCGTGTTTCATTGCTTGATG	
ermG	ATAGGTGCAGGGAAAGGTCA	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35x
	TGGATTGTGGCTAGGAAATGT	
tetQ	ATCGGTATCAATGAGTTGTT	95 °C 15 s, 50 °C 1 min, 72 °C 30 s, 35x
	GACTGATTCTGGAGGAAGTA	
tetX	TTAGCCTTACCAATGGGTGT	95 °C 15 s, 55 °C 30 s, 72 °C 30 s, 35x
	CAAATCTGCTGTTTCATTCG	
tetX1	TCAGGACAAGAAGCAATGAA	95 °C 15 s, 50 °C 1 min, 72 °C 30 s, 32x

	TATTTCGGGGTTGTCAAACT	
bexA	TAGTGGTTGCTGCGATTCTG	95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 35x
	TCAGCGTCTTGGTCTGTGTC	
IS4351	CAGGGTCTGGATACGCAAGT	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35x
	CTGATAAGCCCGTTGGTGTT	
gyrA	CTACGGAATGATGGAACTGG	95 °C 15 s, 53 °C 30 s, 72 °C 30 s, 35x
	TGTTCAGACGTGCTTCAGTG	

Table 3. RT-PCR results of the MDR *Bacteroides species* strains

Strains	***cfiA***	***cfiA* IS**	***cepA***	***cfxA***	***cfxA upstream***	***nim***	***ermF***	***ermF IS***	**IS*4351***	***ermB***	***ermG***	***tetQ***	***tetX***	***tetX1***	***bexA***	***gyrA***
D92	-	N. A.*	-	-	N. A.	-	-	N. A.	-	-	+	+	-	-	-	N. A.
SZ4	-	N. A.	-	+	1.2 kb**	-	-	N. A.	+	-	-	+	-	-	-	N. A.
SZ9	-	N. A.	-	+	D/IS***	-	+	N. A.	-	-	+	+	+	-	+	N. A.
SZ34	-	N. A.	-	-	N. A.	-	+	N. A.	-	-	-	+	+	-	-	N. A.
SZ35	-	N. A.	-	+	D/IS	-	+	-	+	-	-	+	+	-	+	N. A.
SZ38	+	282 bp	-	-	N. A.	-	-	N. A.	-	-	-	+	-	-	-	82Ser→Phe

*N. A.: not applicable **1.2 kb regulator region ***D/IS.: deletion or otherIS-element