**Epidemiological- and antibiotic susceptibility investigations of Hungarian**

*Bacteroides fragilis* **group clinical isolates**

**Theses of doctoral dissertation**

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## I.    INTRODUCTION

### I.1.    *BACTEROIDES* GENUS

In the human body, some organs and surfaces (skin, conjunctiva, oral cavity, upper respiratory, gastrointestinal and urogenital tracts) contain normal microbiota. The human gut microbiota is composed of 500-1000 different species, and 99.9% of these bacteria are obligate anaerobes [1], approximately 25% of the anaerobic bacteria are different *Bacteroides* species [2]. Within the *Bacteroides* genus, the group of the most commonly isolated species is known as the *B. fragilis* group. These bacteria have phisiological functions, but as opportunistic pathogenes can cause severe infections as well. The infections caused by these bacteria are usually polimicrobial, caused by aerobic facultative and obligate anaerobic bacteria and members of *B. fragilis* is found in most of these infections with an associated mortality of more than 19%; however, it accounts only 0.5% of the colon microbiota [2]. The most severe infections caused by *B. fragilis* group isolates are intraabdominal abscesses, gangrenous appendicitis, gynaecological, skin and soft tissue infections, brain abscesses and sepsis [2]. *Bacteroides* species are usually isolated from ovaries, fallopian tubes and Bartholin's abscesses, *B. thetaiotaomicron* can be recovered from pelvic inflammatory disease (PID) [2]. *Bacteroides* strains rarely cause Central Nervous System (CNS) infections (including brain abscess, subdural or epidural empyema, and meningitis), endocarditis and pericarditis [2,3]. Septic arthritis and osteomyelitis are rare, associated with hematogenous spread, prosthetic joint, rheumatoid arthritis or trauma.

### I.2.    IMPORTANCE OF ANTIBIOTIC SUSCEPTIBILITY TESTING AND SURVEILLANCE

The number of the anti-anaerobic antibiotics is quite limited, which are the followings: cephamycins (e.g. cefoxitin), β-lactam/β-lactamase inhibitor combinations, carbapenems, 5-nitroimidazoles, clindamycin, tigecycline, chloramphenicol, fluoroquinolones. The routine antibiotic susceptibility testing among anaerobic bacteria is not recommended by the Clinical and Laboratory Standards Institute (CLSI). In Hungary only a very few studies have been performed so far investigated the antibiotic susceptibility pattern of *B. fragilis* group isolates [4,5]. As the consequence of the extended usage of

antibiotics, not only among aerobic but also the anaerobic bacteria appeared the MDR isolates.

## II.    AIMS OF THE STUDY

Only a few (inter)national studies have been published in the topic of antibiotic susceptibility pattern of *B. fragilis* strains. These studies reported increasing antimicrobial resistance among human clinically relevant *B. fragilis* group isolates in case of some antibiotics and significant geographical difference among these data. The consequence of the widespread antibiotic usage was the appearance of MDR *Bacteroides* isolates; we found firstly the relative significant prevalence of MDR *Bacteroides* strains within a comprehensive study. In Hungary, during the last two decades no comprehensive antimicrobial susceptibility study was performed. Some *B. fragilis* strains can produce enterotoxin, encoded by *bft* gene. The distribution of this gene and its isotypes were investigated approximately one decade ago in Hungary. In the literature very little data can be achived concerning the prevalence or distribution of *bft* gene and its isotypes and their correlation with C10 and C11 cysteine protease (*bfp1-4, fpn)* and *cfiA* genes. Consideration of these facts, our aims were:

1.    Validation of the MALDI-TOF MS method for the identification of different *B. fragilis* group clinically relevant isolates.

2.    Epidemiological investigation of antimicrobial susceptibility testing of 400 clinically relevant *B. fragilis* isolates from different geographical area of Hungary and compare the resistance trends data with international and previous Hungarian data.

3.    Molecular investigation of the antimicrobial resistance genes and other genetic elements of MDR *Bacteroides* isolates found among the 400 *B. fragilis* group clinical isolates.

4.    Determination of the distribution of *B. fragilis* enterotoxin gene (*bft*) and its isotypes and C10 and C11 cysteine protease genes (*bfp1-4, fpn*) of 200 *B. fragilis* strains of the 400 *B. fragilis* group isolates.

## III.    MATERIALS AND METHODS

### III.1.    VALIDATION OF MALDI-TOF MS METHOD

#### III.1.1.    BACTERIAL STRAINS

In our study 400 *B. fragilis* group isolates, collected between 2014 and 2016 by four Hungarian clinical microbiological centres (Centre 1: Semmelweis University, Budapest; Centre 2: SYNLAB Ltd., Budapest; Centre 3: University of Debrecen; Centre 4: University of Szeged) were investigated in this study. The strains (n=10) obtained from the University of Pécs were investigated with the isolates from the centre 1 together. The collection criteria was the isolation of the first 100 clinically relevant, non-repeating samples by each centres. The strains were stored at -80 °C in cryobank vials with Brain Heart Infusion (BHI) medium and with 20% glycerol until use. All the examined strains were cultured on Schaedler agar (bioMérieux, France) for 48 hours, at 37 °C in anaerobic chamber.

#### III.1.2.    MALDI-TOF MS

The strains were identified in each center and also in Szeged with MALDI-TOF MS (Bruker Daltonik, Germany) with Biotyper Version 3.0 software. Results with the best log score values were accepted. A sum of 21 strains with contradictory results of identification and re-identification obtained by MALDI-TOF MS were checked by biochemical test kit of rapid ID 32A method. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29742 were used as control strains. The DNA amplicons from RT-PCR reactions were purified and sequenced. 16S rRNA gene was amplified by RT-PCR and the obtained sequencing data were analyzed by NCBI BLAST and leBiBi softwares.

### III.2.    ANTIMICROBIAL SUSCEPTIBILITY TESTING

The Minimal Inhibitory Concentration (MIC) values for ten antibiotics were determined with the agar dilution method according to the recommendation of the CLSI [6], which were the following: ampicillin, amoxicillin/calvulanic acid, cefoxitin, meropenem, clindamycin, metronidazole, moxifloxacin, tetracycline, tigecycline, chloramphenicol. For the interpretation of the MIC-value, we used the breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing

(EUCAST) or the CLSI [6,7]. As the tigecycline breakpoints among *Bacteroides* species have not yet been established, the breakpoints published by Nagy *et al.* were applied for the interpretation [5]. Here, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 served as control strains.

### III.3.     MOLECULAR INVESTIGATION OF MULTIDRUG RESISTANT STRAINS

Among the 400 *B. fragilis* group isolates we found six MDR isolates. 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* as Eitel *et al*. described earlier [8]. The DNA amplicon of the gyrA gene of SZ38 *B. fragilis* strain was purified and sequenced with ABI BigDye® Terminator Version 3.1 (Thermo Fisher Scientific, USA) kit in the Series Genome Analyser 3500 (Life Technologies, USA).

### III.4.     PCR DETECTION OF *B. FRAGILIS* ENTEROTOXIN GENE AND ITSISOTYPES AND THE C10 AND C11 CYSTEINE PROTEASE GENES

Using the procedure outlined by Soki *et al.*, a RT-PCR was performed for the detection of *bft* gene in the case of all 200 *B. fragilis,* using bftF and bftR primers [9]. For the typing of the *bft* gene, an internal fragment of *bft* gene was amplified and a melting point analysis was performed. These PCR products were purified and investigated with RFLP as well to differentiate the isotypes of the gene. An internal fragment of three *bft-1* and three *bft-2* harbouring *B. fragilis* isolates was sequenced to confirm the possible separation of *bft*-1 and *bft*-2 harbouring isolates based on the melting-point analysis using the RT-PCR. The prevalence of the *bfp1-4* and *fpn* genes of the C10 and C11 proteases, respectively, was investigated in a subset of 26 *bft*-positive and 46 *bft*–negative *B. fragilis* strains by RT-PCR.

## IV.    RESULTS

### IV.1.    VALIDATION OF MALDI-TOF MS METHOD

During the routine identification in local laboratories and re-identification in Szeged, out of 400 strains, 379 (94.75%) were correctly identified to species level with the log score value of ≥2.000 (log score value range: 2.020–2.525, average log score value: 2.249). Among the results of three parallel MALDI-TOF MS re-identification, the best log score from the identification results was chosen. Contradictory MALDI-TOF MS re-identification results of 21 strains (four *B. fragilis* and 17 non-fragilis *Bacteroides*) were confirmed by 16S rRNA gene sequencing method and investigated with rapid ID 32A. The same identification results with MALDI-TOF MS and sequencing were obtained in case of 15 (71.42%; 15/21) isolates. Excellent identification results (>95.0%) were obtained with rapid ID 32A only in case of eight strains (31.01%, 8/21). In comparison of identification results with MALDI-TOF MS and rapid ID 32A, we reported only five concordant results (23.81%, 5/21).

### IV.2.    INVESTIGATION OF ANTIBIOTIC SUSCEPTIBILITY

A total of 98.0% of the strains (were resistant to ampicillin; whilst only 4.5% displayed resistance to amoxicillin/clavulanic acid. The rate of cefoxitin resistant strains was 6.75%; whilst a relatively high resistance rate of 7.0% was found to meropenem. And 36.75% of the isolates displayed high resistance to clindamycin. Metronidazole remained very active against *Bacteroides* species, with only one strain demonstrating resistance (0.25%). The overall resistance rate to moxifloxacin was 18.5%. CLSI breakpoints indicated a high resistance of 65.25% to tetracycline; 94.75% of the isolates were susceptible to tigecycline and no resistance was seen to chloramphenicol. Comparing the cefoxitin resistant rates of the centres, the difference was significant between Centre 3: (3.00%) and Centre 4 (13.00%) (p<0.001). We detected a relatively high difference in meropenem susceptibility data. In Centre 4 we identified 11 *B. fragilis* and one *B. ovatus* meropenem resistant strains. In other centres the rate of meropenem resistant strains was lower (4.0-7.0%). Clindamycin resistance displayed a relatively strong geographical difference, which was significant between Centre 1 (48.0%) and Centre 3 (27.00%) (p=0.003).

**IV.3.      MOLECULAR INVESTIGATION OF MDR *BACTEROIDES* STRAINS**

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. MDR isolates in question displayed resistance to a range of resistance from four to six different antibiotic classes. The *B. fragilis* SZ38 isolate harboured the *cfiA* gene, 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. *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. All of the isolates harboured the *tetQ* gene and three of them expressed a high level tetracycline resistance (MIC≥32 mg/l). None of the isolates harboured the *nim* gene and *B. thetaiotamicron* SZ35 harboured the *bexA* efflux gene. Point mutations were investigated in the case of the *gyrA* gene of the *B. fragilis* SZ38 strain, and with a sequence analysis Ser82Phe substitution in the QRDR region of the GyrA subunit of gyrase enzyme was detected.

**IV.4.      INVESTIGATION OF *B. FRAGILIS* ENTEROTOXIN**
**         AND CYSTEIN PROTEASES GENES**

Out of the 200 *B. fragilis* isolates, 26 (13.0%) turned out to harbour the *bft* gene detected by RT-PCR. Twenty proved to be *bft-1* and six *bft-2* isotypes after performing PCR-RFLP. We did not find any isolate carrying the *bft-3* isotype among the ETBF strains. A good correlation was observed between the results obtained by the melting curve analysis to differentiate *bft*-1 and *bft*-2 and the search for the typical bands by PCR-RFLP to differentiate between the *bft*-1 and *bft*-2 isotypes. To investigate the presence of *bfp1-4* (the C10 protease gene) and *fpn* (the C11 protease gene) a subset of 72 *B. fragilis* isolates (26 ETBF strains and 46 non-ETBF strains) was analysed via RT-PCR. 38 strains harboured *bfp1*, 58 isolates contained *bfp2* gene; while 17 isolates were positive for *bfp3* and no *bfp4* positive strain was detected. Nine strains simultaneously harboured *bfp1*, *bfp2* and *bfp3* genes; 22 were positive for *bfp1* and *bfp2;* while five isolates contained *bfp2* and *bfp3*; and one isolate proved to be positive for *bfp1* and *bfp3*. Among the 24 of the 26 *bft*-positive

strains (92.3%) containing the *fpn* gene; while 36 of the 46 *bft*-negative isolates (78.3%) did harbour the *fpn* gene either. Among the *cfiA*-positive isolates, three harbouring *bfp-1* and two *bfp-3* were identified; while among the *cfiA*-negative strains 35 proved positive for *bfp1*, 56 for *bfp2* and 17 for *bfp3*. A significant negative correlation was demonstrated between *cfiA* and *fpn* (p<0.000) genes.

## V.    DISCUSSION

### V.1.    VALIDTION OF MALDI-TOF MS METHOD

Rapid ID 32A (bioMérieux, France) cannot make difference between gram-negative and gram-positive bacteria ("one fits all"). The database needs always to be improved and expanded with the newly recognized species. Another disadvantage of the biochemical test can be the length of incubation time and different incubation conditions. 16S rRNA gene sequencing is the most accurate method, but it is complicated, time-consuming and expensive features inhibit the application in routine clinical microbiology. The MALDI-TOF MS system revolutionized and simplified the identification of various clinical isolates. This method is easy to perform within a short period of time and reproducible and this has a high discriminatory power. We demonstrated that 94.75% of *Bacteroides* isolates were correctly identified with Biotyper software 3.0. The differences among the results by MALDI-TOF MS and 16S rRNA gene sequencing can be explained with the classification in the same phylogenetical clade of *Bacteroides* strains SY9, SY64, and SY81.

### V.2.    ANTIBIOITIC SUSCEPTIBILITY TESTING

This study confirms previous findings that ampicillin resistance is very high (98.0%) due to the widely distributed *β*-lactamase producing genes among *Bacteroides* isolates. [6]. Only 4.50% of our isolates were resistant to amoxicillin/clavulanic acid, while Nagy *et al.* reported a rate of 8.7% [6]. Some 6.75% of the strains exhibit a resistance to cefoxitin, which is much lower than reported in previous surveys (15.2-17.2%) [6,10]. This study confirmed an overall species resistance level of 7.0% was observed to meropenem. A meropenem resistance rate for the *B. fragilis* group isolates of 0.5% was reported in an American study [11]. We noticed an elevated overall resistance level of 36.75% to

clindamycin, which varied among the different species. Others have reported a clindamycin resistance rate of between 27.0 and 37.6% [5,12]. Only one strain resistant to metronidazole (0.25%), the overall resistance rate to metronidazole among *Bacteroides* isolates remains low (<1%) [5]. We detected an overall resistance rate to tetracycline of 65.25%, tigecycline was very active, with only three resistant strains isolated (0.75%), which is consistent with the results published by Nagy *et al.* (1.7%) [5]. The effectiveness of chloramphenicol remained excellent (no resistant strain was found) and Wybo *et al.* reported a susceptibility of 99% of 2004 [12]. According to the data reported by Nagy *et al.*, the level of clindamycin resistance increased from 23% to 36.75% and moxifloxacin from 13.6% to 18.50%, but interestingly the level of resistance to amoxicillin/clavulanic acid decreased from 15% to 4.5% and cefoxitin from 24% to 6.75% [4,5].

## V.3.      MOLECULAR INVESTIGATION OF MDR *BACTEROIDES* STRAINS

We found six MDR isolates of 400 *Bacteroides* strains, which displayed a 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 by Urbán *et al*., 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 [9].

## V.4.      INVESTIGATION OF *B. FRAGILIS* ENTEROTOXIN
##            AND PROTEASE GENES

Our data showed that the majority of the *bft*-positive isolates contained the *bft-1* allele (76.9%, 20/26)*,* while 23.1% (6/26) contained the *bft-2* allele and there no *bft-3* harbouring strain was detected. Scotto d'Abusco *et al.* investigated intestinal and extraintestinal ETBF strains and reported that the most common isotype was *bft-1* (10/16, 62.5%), while 25.0% (4/16) harboured the *bft-2* isotype and 12.5% (2/16) harboured the *bft-3* isotype [13]. Among the *bft*-positive and –negative *B. fragilis* strains investigated, the *bfp2* gene was the most prevalent and a positive correlation was found between the *bfp2* gene and *fpn* gene. According to our results, amongst the 26 *bft*-positive strains 24 contained the *fpn* gene, which confirms the key role of fragipain in the activation of *B. fragilis* enterotoxin. Nevertheless, 36 *bft*-negative *B. fragilis* isolates also contained the

*fpn* gene. We found quite similar rate of the *bft* harbouring strains (13.0%) compared with our previous study from 2006 (8.7%) [14]. The first study of our Institute reported higher *bft* carriage rate (25.3%), but the strains were investigated not by molecular method but HT29/C1 cell culture [15].

## VI.    CONCLUSIONS

1. We validated the accuracy of MALDI TOF MS method for the clinically relevant *B. fragilis* group isolates. Three measurement with MALDI-TOF MS method and comparison of the results was performed. In case of discrepant results we performed 16S rDNA sequencing and our study proved the high accuracy (94.75%), species-level identification of MALDI TOF MS method among *B. fragilis* group strains. Our study proved the superiority of MALDI-TOF MS system to traditional and automatized biochemical tests.

2. It was the first comprehensive antibiotic susceptibility study in Hungary, performed with uniform criteria and method. We interpreted the antibiotic susceptibility test results and compared them with international and previous Hungarian data. This study confirms that ampicillin resistance is very high, but only 4.5% of isolates were resistant to amoxicillin/clavulanic acid. The rate of cefoxitin, tetracycline and moxifloxacin resistance depends on the different species. High resistance level (7.0%) was observed to meropenem and 8.58% of the strains harboured the *cfiA* gene. We noticed an elevated resistance level of 36.75% to clindamycin, which varied among the different species. Metronidazole, tigecycline and chloramphenicol remained excellent drug of choice.

3. We found significant prevalence of MDR *Bacteroides* strains (six MDR isolates of 400 *Bacteroides* strains) which displayed a resistance to four to six different antibiotic groups. According to the detailed molecular investigation the molecular background of resistance pattern of these MDR isolates differ from strain to strain.

4. We investigated the incidence of *B. fragilis bft* gene from extraintestinal isoaltes; as well as the incidence of C10 and C11 cysteine protease genes and *cfiA* gene together. 13.0% of the strains harboured *bft* gene, which is quite similar to our previous study from 2006 (8.7%). Our data showed that the majority of the isolates contained the *bft-1* allele, while 23.1% contained the *bft-2* allele and there no *bft-3* harbouring strain was detected. We found a *B. fragilis* strain that harboured the *cfiA* and *bft* gene and its isotypes simultaneously, which

is a rare finding. Amongst the *bft*-positive strains 24 contained the *fpn* gene, which confirms the key role fragipain in the activation of *B. fragilis* enterotoxin.

The main conclusion of our survey and our results proved that the periodic monitoring of the antimicrobial susceptibility of *Bacteroides* species is essential to obtain accurate information on local and national rates of antimicrobial resistance, and that this is critical to guide appropriate therapy for patients.

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## LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THIS THESIS

I.     **Károly Péter Sárvári**, József Sóki, Miklós Iván, Cecília Miszti, Krisztina Latkóczy, Szilvia Zsóka Melegh, Edit Urbán: MALDI-TOF MS versus 16S rRNA sequencing: minor discrepancy between tools in identification of *Bacteroides* isolates.
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2017; Sept. 11:1-9 doi: 10.1556/030.64.2017.025.       **IF: 0.921**

II.     **Károly Péter Sárvári**, József Sóki, Katalin Kristóf, Emese Juhász, Cecília Miszti, Krisztina Latkóczy, Szilvia Zsóka Melegh, Edit Urbán: A multicentre survey of antibiotic susceptibility of *Bacteroides* species from Hungary.
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III.     **Károly Péter Sárvári**, József Sóki, Katalin Kristóf, Emese Juhász, Cecília Miszti, Krisztina Latkóczy, Szilvia Zsóka Melegh, Edit Urbán: Molecular characterization of Multidrug Resistant *Bacteroides* isolates from Hungarian clinical samples.
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IV.     **Károly Péter Sárvári**, József Sóki, Miklós Iván, Cecília Miszti, Krisztina Latkóczy, Szilvia Zsóka Melegh, Edit Urbán: Detection of enterotoxin and protease genes among Hungarian clinical *Bacteroides fragilis* isolates.
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I.     **Karoly Peter Sarvari**, Szilard Zolyomi, Gergely Agoston, Gabriella Terhes, Henriette Gavaller, Tamas Forster, Albert Varga, Edit Urban: A rare case of acute myocarditis.
*J Med Microb Diagn*   Open Access 2015, 4:3       **IF: 1.90**

II.     **Karoly Peter Sarvari**, Bela Vasas, Ildiko Kiss, Andrea Lazar, Istvan Horvath, Marianna Simon, Zoltan Peto, Edit Urban: Fatal *Clostridium perfringens* sepsis due to emphysematous gastritis and literature review.
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