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 physiological functions, but as opportunistic pathogens can cause severe infections as well. The infections caused by these bacteria are usually polymicrobial, 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: cephemycins (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 achieved 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.

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 IS4351; while the end-point PCR method were used for the amplification of the upstream region of cfiA, cfxA genes and IS4351 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 ITS ISOTYPES 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 IS4351 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. thetaiotaomicron 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 \textit{fpn} gene; while 36 of the 46 \textit{bft}-negative isolates (78.3%) did harbour the \textit{fpn} gene either. Among the \textit{cfIA}-positive isolates, three harbouring \textit{bfp}-1 and two \textit{bfp}-3 were identified; while among the \textit{cfIA}-negative strains 35 proved positive for \textit{bfp1}, 56 for \textit{bfp2} and 17 for \textit{bfp3}. A significant negative correlation was demonstrated between \textit{cfIA} and \textit{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 \textit{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 \textit{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 \textit{β}-lactamase producing genes among \textit{Bacteroides} isolates. [6]. Only 4.50% of our isolates were resistant to amoxicillin/clavulanic acid, while Nagy \textit{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 \textit{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, harbouring cepA, cfiA, erm, nimA, tetQ genes and IS1187 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 isolates; 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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*IF: 1.119*

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