Phenotypic and genotypic investigation of antibiotic resistance of clinical *Bacteroides* isolates

Theses of doctoral dissertation

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CONTENT

1. INTRODUCTION

1.1. Description of *Bacteroides fragilis* group strains

Species belonging to *Bacteroides fragilis* group are anaerobic, bile-resistant, non-spore-forming and non-motile Gram-negative rod shaped bacteria. *Bacteroides spp.* are abundant anaerobic bacteria in the colon (10^{10}-10^{11} cells per gram of human faeces). They may be passed from mother to child during vaginal birth and thus become part of the human flora in the very early stages of life [Simon and Gorbach, 1984; Reid, 2004]. There are several advantages of the presence of these bacteria in the intestinal tract: they have role in carbohydrate fermentation, produced short chained fatty acids (in cooperation with other intestinal microorganisms), thus ensuring the daily energy and nutrient requirement of the host organization [Xu and Gordon, 2003]. Recent metagenomic studies have confirmed the usefulness of the *Bacteroides* species to the normal human intestine: their reduced presence may result various adverse physiological processes, such as obesity, or inflammations [Wu *et al*., 2004; Ley *et al.*, 2005; 2006**].**

In addition, *Bacteroides* are opportunistic pathogen organisms. Although they represent only 0.5% of the bacterial population occur in the faeces, *B. fragilis* group strains are the most frequently isolated species in anaerobic infections. Practically pathogen *Bacteroides* strains can be isolated from infections of any part of the human body [Finegold, 1995]. They cause severe intra-abdominal infections, postoperative wound, skin and soft-tissue infections together with other anaerobic and aerobic bacteria, or they might also be the causative agents of bacteremia. The most virulent *Bacteroides* species is the *B. fragilis*. This organism is responsible for the 80% of the infections caused by the members of the *Bacteroides* genus and are considered the most virulent strain in *B. fragilis* group strains. The *B. fragilis* is found in many anaerobic infections with an associated mortality of more than 19% in bacteremia and if a documented *B. fragilis* infection is left untreated, the mortality rate is reported to be about 60% [Goldstein, 1996]. Healthy individuals rarely become infected; the infection is usually due to a pre-existing underlying disease or after any intraabdominal surgery procedures. *B. fragilis* and related species possess numerous virulence factors,

antibiotic resistance genes, which enables a high degree of ability to infect the human body. The *Bacteroides* isolates require glucose, haemin, minerals and vitamin B_{12} and are resistant to 20% bile.

In 1898, Veillon and Zuber was the first who described *B. fragilis* as *Bacillus fragilis* [Veillon and Zuber, 1898]. Currently we know more than 20 *Bacteroides* species and 5 *Parabecteroides* species, for example *Parabacteroides distasonis* (formerly *Bacteroides distasonis*), and *Parabacteroides merdae* (formerly *Bacteroides merdae*). [Wexler, 2007].

1.2. The antibiotic resistance of *B. fragilis* group strains

Antibiotic resistance in *Bacteroides* strains can be categorized into three main groups:

1. intrinsic resistance (aminoglycosides, 1st and 2nd generation quinolones, 1st and 2nd generation cephalosporines)
2. increasing resistance (β-lactam antibiotics such as penicillin, ampicillin, as well as erythromycin, tetracycline, clindamycin)
3. low level resistance to the antibiotics recommended for treatment of infections involving *Bacteroides* strains (β-lactam/β-lactamase inhibitor combinations, carbapenems, metronidazole, certain 3rd and 4th generation quinolones)

Bacteroides strains isolated from clinical samples have different levels of resistance to different families of antibiotics. Members of the *Bacteroides* genus are originally resistant to aminoglycosides. The reason of this phenomenon is that the *Bacteroides* strains are anaerobic microorganisms, therefore, they do not have oxygen or nitrate-dependent electron transport system, which would be required for the uptake of these antibiotics [Rasmussen *et al.,* 1993]. In addition, *B. fragilis* group strains has inherent resistance also against the 1st and 2nd generation quinolones [Rasmussen *et al*., 1993] (actually the 3rd and 4th generation quinolones were developed to act on infections involving anaerobic bacteria; and resistance to them has only begun to appear in the past few years) [Sutter and Finegold, 1976].

In case of some β-lactam antibiotics such as penicillin, ampicillin, but also in case of erythromycin, tetracycline and clindamycin a constantly increasing resistance was observed, because of this, the therapeutic usage of these drugs can be recommend only after the antibiotic susceptibility testing of the isolates has been done [Rasmussen *et al*., 1993; Nagy *et al*., 2011]. The carbapenems (imipenem, meropenem), β-lactam/β-lactamase inhibitor combinations (amoxicillin/clavulanic acid, piperacillin/tazobactam), the newer fluoroquinolones (moxifloxacin, trovafloxacin, gemifloxacin) and metronidazole are antibiotics which can most successfully used in empiric therapy of infections involving *B. fragilis* group isolates [Wadsworth-KTL 6th edition, 2002; Löfmark *et al*., 2010; Nagy *et al*., 2011]. The resistance level of *Bacteroides* strains to these antibiotics may vary depending on the geographical location where the strain was isolated [Nagy *et al.,* 2011]. The development of the current rather high resistance values to some antibiotics can be due to extensive and not always appropriate antibiotic usage similar to aerobic and facultative anaerobic bacteria [Rasmussen *et al.,* 1993; Edwards, 1997; Wexler, 2007]. In addition, nowadays increasing number of reports on multi-resistant clinical isolates of *Bacteroides* have been published [Rotimi *et al*., 1999; Wareham *et al*., 2005; Hartmeyer *et al*., 2012].

1.3. The antibiotic susceptibility testing methods for anaerobes

Infections involving anaerobes are usually treated empirically based on published surveillance data. The indications for susceptibility testing for anaerobes are the followings: 1) there are some specific infections from which isolates should be considered for susceptibility testing (such as endocarditis, osteomyelitis, central nervous system infection, refractory or recurrent bacteraemia, joint infection prosthetic device infection, and organism isolated from any normally sterile site of the body; 2) infections not responsive to empiric therapy or infections which require long-term therapy; 3) to determine patterns of susceptibility of selected anaerobic bacteria in a particular hospital or geographic area; 4) to evaluate the activities of the newly developed antibiotics [Wadsworth-KTL, 2002]. The antibiotic resistance patterns of the clinical isolates may have important implication for clinical outcome.

Recently the European Committee on Antimicrobial Susceptibility Testing (EUCAST) started to harmonize the disk diffusion method for antimicrobial susceptibility testing and classification of resistance for aerobic bacteria [http://www.eucast.org/]. With the emergence of reduced susceptibility towards metronidazole and vancomycin, the need for a simple method for antimicrobial susceptibility testing of an important anaerobic pathogen, *C. difficile* has increased. Based on EUCAST methodology the disk diffusion method was started to be evaluated for *C. difficile* by Erikstrup *et al.* (2012). They found an excellent agreement between inhibition zone diameters by disk diffusion and MICs by E-test. Disk diffusion was able to distinguish between the wild type (susceptible) and resistant and intermediate resistant populations and disk diffusion was able to detect reduced susceptibility towards metronidazole and vancomycin of *C. difficile*. They stated that further studies are needed how can be standardized the disk diffusion method for other, relatively rapid growing anaerobes.

2. AIMS OF THE STUDY

The aims of this study were:

- To evaluate the EUCAST disk diffusion method for susceptibility testing of a large number of *Bacteroides* strains by comparing disk diffusion susceptibility testing results with MICs determined by agar dilution or gradient test (E-test) for a wide variety of antibiotics suggested for treatment of anaerobic infections.
- To determine the incidence of clinically important *cfiA* and *nim* genes among 640 *B. fragilis* group strains obtained from different European countries. Beside to test the two most important resistance genes we were also interested in the presence and distribution of the *bft* gene responsible for the toxin production in *Bacteroides* strains and the possible co- existence

of the *bft* and *cfiA* genes among clinical isolates.

- The incidence of a wide variety of other clinically significant antibiotic resistance genes were also tested among a subset of 161 of the previously tested 640 *B. fragilis* group isolates
- To study the co-occurrence of the detected resistance genes, among *B. fragilis* and non-fragilis *Bacteroides* isolates in connection with their resistance to antibiotics.
- To study the antibiotic resistance and the resistance gene content of a recent collection of *B. fragilis* group isolates obtained from Romania (not included in the previous European surveillance)

3. MATERIALS AND METHODS

3.1. Bacterial strains and cultivation

Out of a big collection of different species belonging to *Bacteroides* and *Parabacteroides* genera 640 isolates were used during the different studies described in this thesis. The strains were collected from 13 European countries for an antibiotic resistance surveillance in 2008-2009 [Nagy *et al*., 2011] and maintained in -80^{o}C in the Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary till usage for different studies described here. In addition, there were 53 *B. fragilis* group clinical isolates (36 *B. fragilis*, 7 *B. thetaiotaomicron*, 7 *B. ovatus* and 3 *B. vulgatus*) which were collected in the period of 2010 and 2013 at the Diagnostic Laboratory of the Emergency Department of the County Hospital at Targu-Mures, Romania as this country did not participate in the Europe-wide surveillance.

All isolates were stored in Brain Heart Infusion (BHI) broth with 15% glycerine at -80 °C and were cultivated at 37 ºC anaerobically on Brucella blood agar supplemented with haemin (0.005 g/l) and vitamin K1 (0.01 g/l) (Becton Dickinson, Heidelberg, Germany) in an anaerobic cabinet (Concept 400; Ruskinn Technology Ltd., Bridgend, UK.

3.2. Evaluation of the applicability of the disk diffusion method for the antibiotic resistance determination of *Bacteroides* strains

The inoculum from the 24 h primary plates of the isolates involved in the evaluation of the applicability of the disk distribution test for the antibiotic resistance determination was prepared in physiological saline to reach McFarland 1. The 15-15-15-minute rule of EUCAST was used. Nine antibiotics were tested during the disk diffusion measurements on Brucella blood agar supplemented with haemin and vitamin K1 (Becton Dickinson, Heidelberg, Germany). The antibiotic disks were as follows: amoxicillin/clavulanic acid (20/10 µg/disk), piperacillin/tazobactam (30/6 µg/disk), cefoxitin (30 µg/disk) imipenem/cilastatin (10 µg/disk), meropenem (10 µg/disk), clindamycin (10 µg/disk), tigecycline (15 µg/disk), metronidazole (5 µg/disk), moxifloxacin (5 µg/disk). All disks were obtained from BioRad (Marnesla-Coquette, France) except metronidazole and clindamycin, which were purchased from Oxoid (Basingstoke, UK).

3.3. Detection of genes responsible for antibiotic resistance and enterotoxin production by Real-Time PCR (RT-PCR) method

To detect the various antibiotic resistance genes and *bft* gene the bacterial cells from the surface of 24 h anaerobic agar plates were suspended in 100 µl distilled water in 1.5 ml Eppendorf tubes, and incubated at 100 ºC for 10 min. The supernatants of the centrifuged suspensions (2 min, 14,000 rpm) were used as template DNA and stored at -20 ºC until use. Primers suitable for providing products in RT-PCR experiments, using the known nucleotide sequences of the genes, were designed by the Primer3 software (http://frodo.wi.mit.edu/). Each reaction mixture contained a 5 µl 2x PCR “mastermix” (iQ, Bio-Rad or Brilliant II, Stratagene), 0.7 µM (35 pmoles) of each primer, 1 µl template DNA, 0.5 µl EvaGreen (Biotium) DNA-binding fluorescent dye (for the iQ “mastermix”) dye and sterile water up to 10 µl final volumes in plastic PCR plates. Amplification was performed in MxPro3000 (Stratagene, USA) or StepOne (Life-Technologies) Real-Time PCR instruments. The amplification and the melting curves

were observed at a wavelength of 415 nm required for the SYBR Green and the EVA Green dyes. The initial denaturation by the amplification cycles was 10 min (iQ) or 5 min (Brilliant II). Positive reactions were identified by the starting amplification cycle, melting curves showing the correct melting temperatures, and in rare cases where it was required to compare the size of the products with those of the positive controls in 1.2% agarose gel electrophoresis. Nucleotide sequencing of the *tetX1* (*B. fragilis* BM13) and *linA* (*B. fragilis* TR23) was carried out as described previously [Brisson-Noël and Courvalin, 1986; Whittle *et al*., 2001], and their sequences were compared to the reference sequences (*linAn2* AF251288 and *tetX1* AJ311171) by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4. PCR RFLP for investigation of *bft* gene alleles

The *bft* alleles of the *bft* gene positive strains obtained by the RT-PCR were determined by PCR-RFLP. Using the BTT1 (CATGTTCTAATGAAGCTGATTC) and BTT2 (ATCGCCATCTGCTGTTTCCC) primers the entire *bft* genes were amplified in end-point PCRs (95 ºC 10 min 1x; 95 ºC 30 sec, 62 ºC 1 min, 72 ºC 1 min, 35x). The PCR products were purified with the HighPure PCR Cleanup Kit (Roche Diagnostics GmbH, Mannheim, Germany) after agarose gel electrophoresis and the pure products were digested with *Mbo*I restriction enzyme. The final products were analyzed by 1.5% agarose gel electrophoresis in TBE buffer, using 0.5 µg/ml ethidium bromide and UV visualization.

3.5. Investigation of the presence of IS elements and their mapping before the resistance genes

PCR templates and reaction setups were the same as described previously, and the PCR strategy to detect resistance genes associated IS elements was also the same as described by Sóki *et al*., 2004; 2006. PCR products, and total DNA samples were electrophoresed in 0.7–1.5% agarose gels in TAE (40 mM Tris-acetate and 1 mM EDTA) or TBE (45 mM Tris-borate and 1 mM EDTA) buffer containing 0.5 µg/ml ethidium bromide; DNA was visualized with UV light, and permanent records were

made electronically.

3.6. Detection of plasmids

The cultivated bacterial cell mass was processed with the Qiagen Plasmid Mini Preparation Kit (Qiagen, Hilden, Germany). Plasmid (200–300 ng) samples were electrophorized in 0.7 % agarose gels containing 0.5 μg/ml ethidium bromide in TAE or TBE buffer with a constant voltage gradient of 5 V/cm.

3.7. Statistical evaluation

Comparisons of the prevalence of different genes in different sets of strains were made by applying chi-squared or Fischer's exact tests with the Sigmaplot 12.0 program (Systat Software, Inc.). The significance threshold level was set at 0.05.

4. RESULTS

4.1. Evaluation of disk diffusion method for antibiotic susceptibility testing of *B. fragilis* group isolates

In this study 381 *B. fragilis* group clinical isolates were involved. from the same collection of isolates used for the antibiotic resistance surveillance during the Europe-wide study. As reference strains *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used, recommended by CLSI for the antibiotic resistance determination by agar dilution.

In the case of imipenem only four isolates were resistant (MIC >8 μg/ml) and two isolates showed intermediate susceptibility (MIC 4-8 μg/ml). For each MIC value the inhibition zones varied from 0 to 13 mm, with 90% of the values within 8 mm. However, the resistant isolates were clearly separated from the susceptible strains: inhibition zone diameter for the resistant strains was ≤ 20 mm and for the susceptible strains ≥ 29 mm, respectively.

The same was true for metronidazole where only 2 isolates were found, which were resistant according to the EUCAST breakpoints, with an MIC 8 μg/ml. All but

one of the susceptible isolates had an inhibition zone ≥ 24 mm. The inhibition zones for the different MIC values varied between 0 and 14 mm and 90% of the values were within 6 mm.

According to the MIC data no amoxicillin/clavulanic acid resistant isolate was among the strains tested. A very large distribution of the inhibition zones of the strains with the same MICs was seen (8-14 mm), however intermediate resistant strains (MIC 8 μg/ml) had an inhibition zone ≤22 mm with some overlap with susceptible isolates.

In the case of piperacillin/tazobactam, three resistant strains were found clearly separated from the intermediate and susceptible population by an inhibition zone ≤16 mm, however intermediate and susceptible isolates overlapped by the disk diffusion method. According to these data, isolates with an inhibition zone ≤24 mm and >16 mm should be tested by the E-test to determine MICs and differentiate fully susceptible from intermediate susceptible isolates. The distribution of inhibition zone diameters for each MIC values varied between 0 and 17 mm.

For testing clindamycin susceptibility of the *Bacteroides* strains by disk diffusion the 10 μg/disk was obtained instead of the 2 μg/disk, used for the antibiotic susceptibility testing of aerobic bacteria. This clearly separated the resistant population with an inhibition zone ≤13 mm. The results showed that the inhibition zone diameters for the clindamycin susceptible strains stretched out between 14 and 42 mm, however the very susceptible (MIC ≤0.125 μg/ml) isolates had a larger inhibition zone diameter in average than those having higher MICs. The distribution of inhibition zone diameters for each MIC values varied between 0 and 23 mm.

In the case of cefoxitin only CLSI breakpoints are available. According to those the susceptible isolates had inhibition zone diameters between 18 mm and 36 mm. The isolates with intermediate MICs (32 μg/ml) had inhibition zone between 18 mm and 27 mm, but this range overlapped with the fully susceptible isolates (MIC ≤16 μg/ml). However, the resistant population (MIC >32 μg/ml) was separated from the susceptible isolates with an inhibition zone ≤15 mm. The zone diameters varied for the different MICs between 0 and 15 mm.

For moxifloxacin only CLSI breakpoints are available. The disk diffusion test clearly separated the susceptible isolates with an inhibition zone ≥19 mm. Only few

isolates were found in the intermediate range with an inhibition zone between 11 mm and 18 mm. All the resistant isolates (MIC >4 μg/ml) had a zone diameter ≤10 mm. The zone diameters varied for the different MICs between 0 and 11 mm with 95% within 6 mm.

For tigecycline no MIC breakpoints are available in the EUCAST or CLSI documents, accordingly only comparison of the MICs and the zone diameters was possible. Among the strains tested only three were found which could be considered fully resistant and had no inhibition zone at all with MICs ≥32 μg/ml. All the strains which had MICs ≤4 μg/ml could be separated with a zone diameter ≥20 mm. For each MIC value the inhibition zones varied from 0 to 11 mm, with 90% of the values within 6 mm.

For meropenem we did not have MIC data from the previous European surveillance study. Most of the strains had a zone diameter ≥28 mm, which separated the few isolates, which can be considered intermediate susceptible or resistant to carbapenems. If we compared the distribution of the zone diameters of imipenem and meropenem more isolates not belonging to the wild type strains (being fully susceptible) could be detected by the meropenem disk.

According to these data, with one exception (cefoxitin), we could suggest tentative zone diameter breakpoints using the disk diffusion method for susceptible isolates of *B. fragilis* group strains based on the MIC breakpoints set by EUCAST and for some antibiotics by CLSI.

4.2. The prevalence of the *cfiA* and *nim* genes among 640 clinical *Bacteroides* isolates originated from Europe and investigation of the IS elements activating these genes

In this part of the study we were interested in the prevalence of the two most important resistance genes among a large cohort of the *Bacteroides* isolates originated from all over Europe. Out of the 640 *Bacteroides fragilis* groups strains 43 (6.7%) harbored the *cfiA* gene and 3 (0.5%) was *nim* gene positive. All the *cfiA* positive isolates belonged to *B. fragilis* giving an 8.8% positivity of the 486 isolates belonging to this species. Out of the 43 *cfiA* positive *B. fragilis* strains 33 proved to be imipenem

sensitive during the MIC determination with MIC <4 μg/ml (data not shown), which shows the wider carriage rate of this resistance gene among the *B. fragilis* clinical isolates than the expression of the carbapenem resistance. From the 640 *Bacteroides* isolates examined 22 had an imipenem MICs ≥4 μg/ml (non susceptible) and out of these 7 isolates had an MIC ≥16 μg/ml (belonging to the resistant category). Out of the 22 imipenem non-susceptible isolates (MICs ≥4 μg/ml) 10 harbored the *cfiA* gene. Of the 10 *B. fragilis* strains with elevated imipenem MICs (4–8 μg/ml) four (40.0%) were *cfiA*-positive, while 6 (85.7%) of the 7 imipenem-resistant (MIC ≥16 μg/ml) *B. fragilis* isolates were *cfiA*-positive. No non-fragilis *Bacteroides* strains were resistant to imipenem. A *cfiA*-negative, but imipenem-resistant *B. fragilis* isolate was identified in this study (*B. fragilis* FI37) with a possible other resistance mechanism than *cfiA*-mediated carbapenemase activity. Among the strains with MIC 4 μg/ml to imipenem and harboring the *cfiA* gene (*B. fragilis* IT15) an IS element has been shown upstream of the *cfiA* gene (IS4351) by PCR mapping. Among the *cfiA*-positive and imipenem-resistant strains (MIC ≥16 μg/ml) four harbored IS elements upstream of the resistance gene. The remaining two *cfiA*-positive isolates that were imipenem-resistant, but without activating IS elements upstream of *cfiA* displayed a heterogeneous resistance phenotype shown by the imipenem E-test. The types of *cfiA*-activating IS elements were IS1187 (n=2), IS614B (n=1), and a novel IS element (n=ISBf11; GenBank accession no. GQ449386) was also described for *B. fragilis* NLH3 that had 77% homology compared with IS614B. *B. fragilis* IT15 harbored IS*4351* upstream of the *cfiA* gene, but its imipenem MIC was lower (4 μg/ml) (intermediate resistant). Two highly imipenem-resistant strains (*B. fragilis* HU61 and FR41) were also genetically "silent", their *cfiA* genes not being activated by IS elements. This phenomenon can be explained by activation of the *cfiA* genes till yet unidentified mechanism that boosts the carbapenemase activity of the strains.

Of the 640 *Bacteroides* strains, 21 had reduced susceptibility to metronidazole (MIC ≥ 4 μg/ml) and only 3 (*B. fragilis* IT724 and IT797 and *B. thetaiotaomicron* HU66) harbored *nim* genes, with the following metronidazole MICs 0.125 μg/ml (*B. fragilis* IT797), 1 μg/ml (*B. fragilis* IT724) and 256 μg/ml (*B. thetaiotaomicron* HU66). An examination of the *nim*-mediated resistance mechanisms revealed that *B. fragilis*

IT797 and IT724 harbored chromosomal *nimA* and *nimC* genes, respectively. By contrast, the *nimE* gene of *B. thetaiotaomicron* HU66 was located on an 8.3 kb (pBF388c-like) plasmid described earlier [Sóki *et al*., 2006] and was activated by ISBf6. No *nim*-specific plasmids were detected in the two other strains. Furthermore, *B. fragilis* IT797 harbored IS1168 and IS1170, but these elements could not be mapped before the *nimA* gene by PCR mapping. The *nim*-negative but metronidazole-resistant *Bacteroides* strains found in the current study may have other resistance mechanisms (reduced uptake, nitroreductase and pyruvate–ferredoxin oxidoreductase activities, increased lactate dehydrogenase activity, or mutations that alter the carbohydrate utilization affecting the redox state) which shortcut the detrimental cellular effects of this drug.

4.3. Investigation of the prevalence of the *bft* gene among the isolates and determination of the *bft* alleles

Among the 640 *Bacteroides* strains studied, 68 were *bft*-positive (10.6%). All *bft* positive strains belonged to *B. fragilis* providing a 14.0% of prevalence among these isolates. During the PCR RFLP analysis we found that 51 (75.0%) carried the *bft1* allele, 15 (22.1%) carried the *bft2* allele and 2 (2.9%) carried the *bft3* allele. To explore the roles of these alleles in non-intestinal pathogenesis of *B. fragilis*, we checked the distribution of the three *bft* types in isolates originating from different clinical samples, especially among blood culture isolates. The overall *bft* prevalence and the prevalence of the *bft1-3* alleles among the strains examined in this study were not significantly elevated among the blood culture isolates (9.2% vs. 7.3%), but the number of isolates obtained from blood cultures was rather low (n=5), which could be the cause of the non-significant test result (data not shown in detail).

4.4. The prevalence of other antibiotic resistance genes among a subset of the 640 *B. fragilis* group strains

In this study, a more detailed molecular analysis was performed to learn more about the incidence and distribution of the different resistance genes already described

to be present among *B. fragilis* group strains. Out of the 640 strains which were tested for the presence of the *cfiA* and *nim* gene, we chose 161 (128 *B. fragilis* and 33 non-fragilis *Bacteroides*) strains in order to detect the occurrence of the following further genes: *cepA, cfxA, ermB, ermF, ermG, linA, mefA, msrSA, tetM, tetQ, tetX, tetX1*, *tet36* and *bexA*. The selection criteria of the strains were intended to represent the whole collection, taking into account how many strains were collected by the different countries originally and in particular one country (Hungary). The most prevalent resistance genes were *tetQ* (80.1%), *cepA* (70.2%), *ermF* (24.2%) and *linA* (21.7%) with no significant difference among the different European countries. No *nim, tetM* and *tet36* gene was detected among these 161 isolates.

We also compared the prevalence of the tested genes among the *B. fragilis* isolates (128) and those which belonged to different other species of the genus *Bacteroides* (33). *CfiA, ermB, ermG* and *msrSA* were only detected in *B. fragilis* isolates, however no significant other differences were observed in the prevalence of the other genes among *B. fragilis* and non-fragilis isolates.

4.4.1. Correlation of the *cepA*, *cfxA* and *cfiA* genes with the ampicillin, cefoxitin and imipenem resistance among *B. fragilis* and non-fragilis *Bacteroides* strains

All the *B. fragilis* strains (128) were resistant to ampicillin (MIC $\geq$ 2 µg/ml) and 101 of them (78.9%) harbored the *cepA* gene. Among the 33 non-fragilis *Bacteroides* strains which were also resistant to ampicillin, only 12 of them (36.4%), carried the *cepA* gene.

The *cepA* gene distributed with significantly different frequencies among *B. fragilis* and non-fragilis *Bacteroides* strains ($p < 0.001$). The presence of the *cepA* gene did not correlate with the ampicillin MIC values of the tested strains; rather, it occurred among all ampicillin MIC ranges (from 2 to 256 µg/ml). Out of the 11 cefoxitin-resistant *B. fragilis* strains, 3 of them (27.3%) harbored the *cfxA* gene and out of the 9 cefoxitin resistant non-fragilis *Bacteroides* strains just 1 (11.1%) harbored the *cfxA* gene.

The relationship between the cefoxitin MIC values of the strains and the carrying

of the *cfxA* gene is depicted in**.** Interestingly, among the strains with high MIC values (64 μg/ml), the *cfxA* gene was often absent. Also, in contrast to *cepA*, this gene was more common among non-fragilis *Bacteroides* strains (p=0.039).

4.4.2. Correlation of the *ermB, ermF, ermG linA, mefA* and *msrSA* genes with the clindamycin resistance among *B. fragilis* and non-fragilis *Bacteroides* strains

Of the 161 *Bacteroides* strains tested, 40 (24.8%) were resistant (MIC ≥8 μg/ml) to clindamycin. These consisted of 31 (24.2%) *B. fragilis* and 9 (27.3%) non-fragilis *Bacteroides* isolates. The prevalence of the *ermF*, *linA*, *mefA*, *ermG*, *msrSA* and *ermB* genes among all *Bacteroides* strains tested were 39 (24.2%), 35 (21.7%), 20 (12.4%), 9 (5.6%), 9 (5.6%) and 1 (0.6%), respectively. The prevalence of the *ermF*, *linA*, *mefA*, *ermG*, *msrSA* and *ermB* resistance genes among the clindamycin-resistant *Bacteroides* strains were much higher 30 (75.0%), 14 (35.0%), 11 (27.5%), 9 (22.5%), 9 (22.5%) and 1 (2.5%) respectively.

The most common resistance gene was *ermF*, accounting for most of the clindamycin resistant strains. The *ermF* gene was present in 23 (74.2%) of the 31 clindamycin-resistant *B. fragilis* strains tested and 7 (77.8%) of the 9 clindamycin-resistant non-fragilis *Bacteroides* isolates tested. The incidence of the *ermF* gene was almost identical in the *B. fragilis* and non-fragilis *Bacteroides* strains, regardless of whether they were resistant to clindamycin or not. The *msrSA*-positive and the *ermG*-positive isolates harbored at least one other resistance gene and some isolates simultaneously harbored several types of clindamycin resistance genes.

4.4.3. Correlation of the *tetM, tetQ, tetX, tetX1* and *tet36* genes with the tigecycline resistance among *B. fragilis* and non-fragilis *Bacteroides* strains

The prevalence of the *tetQ*, *tetX* and *tetX1* genes among 161 *Bacteroides* strains were 129 (80.1%), 16 (9.9%) and 8 (5.0%), respectively. There were no *tetM*-positive or *tet36*-positive strain among the *Bacteroides* strains tested. Only 3 (1.9%) *B. fragilis* strains were resistant to tigecycline (MIC 16 μg/ml) and all of them carried the *tetQ* gene. Moreover, the *tetM*, *tetX*, *tetX1* and *tet36* genes were not present in any of the

tigecycline-resistant *Bacteroides* strains (data not shown).

4.4.4. Correlation of the *bexA* gene with the moxifloxacin resistance among *B. fragilis* and non-fragilis *Bacteroides* strains

The *bexA* gene, which was considered to be responsible for the moxifloxacin resistance, was present in 12 (7.5%) of the 161 *Bacteroides* isolates tested. These consisted of 6 (4.7%) *B. fragilis* strains and 6 (18.2%) non-fragilis *Bacteroides* strains. This difference is statistically significant (p=0.024). There were no *bexA*-positive strains among the 18 moxifloxacin-resistant *B. fragilis* isolates, and of the 6 moxifloxacin resistant non-fragilis *Bacteroides*, only one (16.7%) harbored the *bexA* gene.

4.5. Antibiotic susceptibility of the *B. fragilis* group strains isolated in Romania and the detection of antibiotic resistance genes

Romania was not part of the *Bacteroides* antibiotic resistance surveillance carried out in 2008-2009, because of this 53 isolates (36 *B. fragilis* and 17 non-fragilis *Bacteroides*) were tested and the data were compared with the data of the Europe-wide study. Despite of the fact that only small number of strains were tested, all the antibiotic resistance tendencies observed during the Europe-wide study could be detected.

Resistance to ampicillin was 96.3%, and 54.7 % of the resistant strains carried the *cepA* gene**.** Among the 8 cefoxitin-resistant isolates only 2 harbored the resistance gene, the *cfxA*. Resistance to amoxicillin/clavulanic acid was 13.0%. 73.0% of the *B. fragilis* strains and 82.4% of the non-fragilis *Bacteroides* were resistant to tetracycline. The resistant strains 78.0% (32) harbored the *tetQ* gene. No imipenem- and metronidazole-resistant isolates were found, however 3 *B. fragilis* strains harbored the *cfiA* gene silently. 11 (20.4%) isolates were resistant to clindamycin. The occurrence of the relevant resistance gene of clindamycin of all the tested 55 strains was the following: 4 *ermF*, 3 *linA*, 2 *msrSA* and 1 *ermB*, respectively. 100% of the *ermF*-positiv strains (4) were clindamycin-resistant. Resistance to moxifloxacin was 13.5% of the *B.*

fragilis isolates and 17.6% of the non-fragilis *Bacteroides* isolates. 9 *bexA*-postive isolates were detected, all of these strains were susceptible of moxifloxacin.

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5. CONCLUSIONS

5.1. Our study confirmed the applicability of the disk diffusion method to distinguish wild- type *Bacteroides* strains from those which are not fully susceptible to drugs usually applied for treatment of anaerobic infections using a large selection of clinical isolates from all over Europe. We managed to set susceptibility breakpoints for inhibition zone diameters for all antibiotics tested except cefoxitin, if we followed strictly the EUCAST rules and we standardized media, inoculum, antibiotic disks' content, incubation time and also the strict anaerobic environment. We found a good agreement between the inhibition zone diameters and the MICs for clindamycin, imipenem, metronidazole, moxifloxacin and tigecyclin. The inhibition zone diameters of meropenem also separated clearly the isolates, which can be considered wild-type isolates. In case of amoxicillin/clavulanic acid and piperacillin/ tazobactam intermediate and susceptible isolates overlap during the zone diameter determination according to the MIC data which did not prevent to separate resistant population.

5.2. Data described in this thesis have provided a fairly complete picture about the occurrence of the *cfiA*, *nim* and *bft* genes among the largest collection of *B. fragilis* group strains tested so fare in Europe (altogether 640 isolates) giving also the possibility to evaluate the genetic background of the measured elevated MICs, observed for imipenem and metronidazole. We could also analyze the rare occurrence of *cfiA-bft* double positive *B. fragilis* isolates originating from four different European countries, showing the possible of more widely spread of this virulent clone of *Bacteroides*. Of the 640 Bacteroides strains only 3 harbored *nim* genes confirming the presence of other resistance mechanisms behind elevated MIC for metronidazole. Of the 22 strains with elevated imipenem MICs (≥4 μg/mL), 10 were *cfiA*-positive and out of these 5 carried also the activating IS elements in the upstream regions of the *cfiA* genes. Other mechanisms behind the elevated MICs for imipenem should also be

presumed.

5.3. Furthermore, the study reported here has provided data concerning the comparison of the antibiotic resistance levels and the presence of different antibiotic resistance genes responsible for their development in Europe using the largest collection of clinical isolates of this genus (214 isolates from 10 European countries, including Romania).

In the case of certain genes (*cepA*, *cfxA*, *cfiA*), our data confirmed earlier findings: (a) *cepA* is very frequent among *Bacteroides* and can be found among non-fragilis *Bacteroides* too, (b) the prevalence of *cfxA* is around 15-20% (in our study 16%) and it is not the only factor for cefoxitin resistance, and (c) *cfiA* has a frequency around 5-7% (in our study 7%) in *B. fragilis* strains. The study also clarified the dominance of some genes (*ermF* and *tetQ*) behind the clindamycin and tigecyclin resistance, respectively. Some additional, known resistance genes such as *tetX*, *tetX1*, *tetM*, *ermB*, *ermG*, *msrSA*, *mefA* and *linA*, could also be detected both among *B. fragilis* and non-fragilis *Bacteroides* clinical isolates.

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Publication related to the Thesis:

I. Sóki J, Eitel Z, Urbán E, Nagy E on behalf of ESCMID Study Group on Anaerobic Infections: Molecular analysis of the carbapenem and metronidazole resistance mechanisms of *Bacteroides* strains reported in a Europe-wide antibiotic resistance survey. International Journal of Antimicrobial Agents, 41:122–125, 2013. (IF: 4.26)

II. Sóki J, Eitel Z, Terhes G, Nagy E, Urbán E on ESCMID Study Group on Anaerobic Infections: Occurrence and analysis of rare *cfiA-bft* doubly positive *Bacteroides* fragilis strains. Anaerobe, 23:70-73, 2013. (IF: 2.36)

III. Eitel Z, Sóki J, Urbán E, Nagy E on behalf of ESCMID Study Group on Anaerobic Infection: The prevalence of antibiotic resistance genes in *Bacteroides fragilis* group strains isolated in different European countries. Anaerobe, 21:43–49, 2013. (IF: 2.36)

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Declaration of co-authorship

Hereby I declare that I have read and approved the thesis submitted for the degree of doctor of philosophy written by Zsuzsa Eitel.
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I have not used them for PhD degree, and I do not attend to do that in the future.

Date: 7 November 2016 Prof. Dr. Elisabeth Nagy

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