Epidemiological- and antibiotic susceptibility investigation of Hungarian *Bacteroides fragilis* group clinical isolates

PhD Thesis

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ABBREVIATIONS

AmpC	AmpC *β*-lactamase
BfPAI	*B. fragilis* pathogenicity islet
BFT	*Bacteroides fragilis* enterotoxin
CA	Conjugative Apparatus
CD4	Cluster of Differentiation 4
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CTn	Conjugative transposon
Em^r	Erythromycin resistance gene
ETBF	Enterotoxin producing *B. fragilis*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GALT	Gut-Associated Lymphoid Tissue
GLC	Gas-Liquid Chromatography
HGT	Horizontal Gene Transfer
IBD	Inflammatory Bowel Disease
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MALDI-TOF MS	Matrix Assissted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry
MBL	Metallo-*β*-lactamase
MFS	Major Facilitator Superfamily
MLS$_B$	Macrolide-Lincosamide-Streptogramin B group
MTn	Mobilizable transposon
NBU	Nonreplicating *Bacteroides* Unit
Nm^r	5-nitroimidazole resistance plasmid
Omp	Outer membrane protein
PBP	Penicilline Binding Proteins
PFOR	Pyruvate:ferredoxine-oxydoreductase enzyme
QRDR	Quinolone Resistance-Determining Region
RND	Resistance Nodulation Division efflux pump family
Tc^r	Tetracycline resistance element
Th2	T helper 2 cell

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. According to modern investigations the human gut microbiota contains 500-1000 different species, and 99.9% of these bacteria are obligate anaerobes [1]. The density of these bacteria increases from the proximal small intestine (10^3 Colony Forming Unit (CFU)/ml) to the colon (10^{11} CFU/g faeces) [1]. The normal microbiota gives several benefits to the host, for example strengthening gut integrity, harvesting energy, protecting against pathogens and regulating host immunity, biotransformation of conjugated bile acids and synthesis of certain vitamins (e.g. vitamin K_2 and different vitamin B-s) [1-3]. Approximately 25% of the anaerobic bacteria are different *Bacteroides* species [4], in the past decades the taxonomy of *Bacteroides* genus has undergone significant changes: some *Bacteroides* species have been placed into genera *Porphyromonas* and *Prevotella* [5]. *B. gracilis* and *B. urealyticus* were moved to the genus *Campylobacter*, while *B. goldsteinii, B. distasonis, B. merdae* were renamed as *Parabacteroides* [4,5]. Other genera have been described for previous *Bacteroides* species (e.g. *Anaerorhabdus*, *Dichelobacter*, *Dialister*, *Fibrobacter*, *Megamonas*, *Mitsuokella*, *Rikenella*, *Sebaldella*, *Tannerella*, *Tissierella* and *Alistipes*) [4]. Within the *Bacteroides* genus, the group of the most commonly isolated species is known as the *B. fragilis* group (Table 1) [4].

Table 1.: Members of the *B. fragilis* group

Bacteroides				***Parabacteroides***
B. acidifaciens	*B. dorei*	*B. heparinolyticus*	*B. salanitronis*	*P. chartae*
B. barnesiae	*B. eggerthii*	*B. intestinalis*	*B. salyersiae*	*P. distasonis*
B. caccae	*B. faecis*	*B. massiliensis*	*B. sartorii*	*P. goldsteinii*
B. cellulosilyticus	*B. finegoldii*	*B. nordii*	*B. stercoris*	*P. gordonii*
B. chinchillae	*B. fluxus*	*B. oleiciplenus*	*B. thetaiotamicron*	*P. johnsonii*
B. clarus	*B. fragilis*	*B. ovatus*	*B. uniformis*	*P. merdae*
B. coagulans	*B. galacturonicus*	*B. plebeius*	*B. vulgatus*	
B. coprocola	*B. gallinarium*	*B. propionifaciens*	*B. xylanisolvens*	
B. coprophilus	*B. graminisolvens*	*B. pyogenes*	*B. xylanolyticus*	
B. coprosuis	*B. helcogenes*	*B. rodentium*	*B. zoogleoformans*	

The *Bacteroides* cells are gram-negative, obligately anaerobic, non-sporeforming, non-motile, rod-shaped, bile-resistant, approximately 0.5 to 1.3 μm wide and 1.6 to 11 μm long rods [6]. Their colonies on an anaerobic blood agar plate (Schaedler-based) are 2-3 mm in

diamater, circular, entire, convex and grey to white in colour. The *B. fragilis* group isolates hydrolize esculin, blackening the BBE (Bacteroides Bile Esculin) agar except for the majority of the *B. vulgatus*, which are esculine-negative. For the presumptive identification of the *Bacteroides* isolates colony morphology, gram-stained smear, traditional biochemical tests (e.g. catalase, oxidase, esculine hidolysis etc.) and identification discs ((e.g. vancomycin (5 μg), kanamycin (1000 μg), colistin (10 μg)) can be applied [6]. These tests are simple, rapid, easy-to-perform and assistant-friendly; however, they are used as orientation, and now unable for exact, reliable, species-level identification, especially for the members of new species. Investigation of the spectrum of produced volatile and non-volatile carbonic acids by gas-liquid chromatography (GLC) is outdated. The traditional-automated methods based on biochemical profile of the bacteria (rapid ID 32A, API 20 A /bioMérieux, France/, Remel rapid ID ANA II /Thermo Fisher Scientific, USA/) have some limitations, e.g. the discrimination ability of biochemically similar strains is not sufficient. The results of identification may depend on the proper anaerobic environment and the deposited species in the library. Some tests (e.g. rapid ID 32A), cannot make difference between gram-negative and gram-positive bacteria ("one fits all"). The database always needs to be improved and expanded with the newly recognized species; other features of these tests are different incubation conditions, depending on the kind and the principle of kits. 16S rRNA gene sequencing is the most accurate method, but needs special equipment and experience, its complicated, time-consuming and expensive features inhibit the application in routine clinical microbiology. The identification of *B. fragilis* group isolates has been revolutionaized by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The principle based on the analysing of the mass:charge ration of the proteins, especially the stable ribosomal proteins of the cells and comparison of their spectra with database of reference strains. In comparison with traditional biochemical tests, the using of MALDI-TOF MS is much easier and provides more accurate identification results of the phenotypically very similar *Bacteroides* strains. Another advantage is that only small amount of biomass is enough, which is very favourable bacause of the relatively slowly growth of the anarobic bacteria. This is a very rapid method: to perform the sample preparation, maesurement and analysis by computer takes only approximately 30 min [7,8]. A further advantage of the MALDI-TOF MS method is that with a special software the *B. fragilis* strains can be separated into two Divisions: Division I (negative for *cfiA* carbapenemase gene) and Division II (harbouring *cfiA* gene) [9]. The database is regularly updated and expended to be able to identify the stains accurately.

1.2. *BACTEROIDES* SPECIES AS MEMBERS OF MICFOBIOME

Bacteroides species has many benefits to the human host: carbonhydrate metabolism, energy production, maturation of Gut-Associated Lymphoid Tissue (GALT), antiallergic effect, colonizing resistance, production of vitamins. The metabolits of the fermentation of polysacchacharids, the volatile fatty acids can be reabsorbed through the large intestine and provide energy for the human host [4]. *B. thetaiotaomicron* can metabolize glycans (e.g. chondroitin sulfate, mucin, hyaluronate, and heparin) sialic acids, hexoseamins, different polysaccharides [10]. *B. fragilis* is able to utilize glycolipids and glycoproteins, as well as mono- and oligosaccharids (galactose, mannose) and complex compounds (*N*-acetyl-*D*-glucosamine, *N*-acetyl-neuraminic acid) [4]. The microbiota is very important in the maturation of GALT, but in the absence of bacteria, the development is defective [11]. Polysaccharid A (PS-A) and B (PS-B) expressed by *B. fragilis* are able to activate CD4+ T lymphocytes. These stimulated cells produce interleukin-10 (IL-10), which prevents other inflammatory responses. The polysaccharides can also activate B cells and promote immunoglobulin M (IgM) production [12]. Some studies hypothesized the important role in the prevention of allergy. Antibiotic and desinfectant usage can damage the microbiota, which leads to the overproduction of T helper 2 (Th2) cytokines and immunoglobulin E (IgE) [13]. The pathogenesis is not clear, PS-A or lipopolisaccharid (LPS) may be involved; low level of LPS can induce the production of IL-12 and interferon gamma (IFN-γ) and decrease the production of Th2 inflammatory cytokines (IL-4, IL-5, IL-13) [4]. The microbiota plays important role in the host defence against the pathogenes. The Paneth cells can be stimulated by *B. thetaiotaomicron* to produce antibiotic proteins, the angiogenin-4 (Ang4) against *L. monocytogenes* [14]. *B. thetaiotaomicron* induce Paneth cells to produce RegIIIγ bactericidal lektin, which can bind to the peptidoglycan of gram-positive bacteria [15]. *Bacteroides* species can inhibit the *Clostridium difficile*-associated infection as well [16]. *Bacteroides* play important role in the biotransformation of conjugated bile acids [4] and produce vitamin K_2 and different vitamin B-s [2,3]. Turnbaugh *et al.* demonstrated, that the presence of *Bacteroides* species is able to prevent obesity [17].

I.3. VIRULENCE FACTORS AND STRUCTURAL ELEMENTS OF *BACTEROIDES* SPECIES

B. fragilis is the most commonly isolated anaerobic pathogen and pocesses the widest range of virulence factors among *Bacteroides* species and can cause even severe infections with

high mortality; however, it accounts only 0.5% of the colon microbiota [4]. *B. fragilis* pocesses different virulence factors: capsule, enzymes, enterotoxin, LPS, adhesive molecules, outer membrane proteins, evasion of host immune response, defense against oxidative stress. *B. fragilis* capsule contains two different high-molecular-weight, antigenetically diverse polysaccharides (polyasaccharid A and B) [4]. PS-A is made up of repeating tetrasaccharide, PS-B repeating hexasaccharide units [4], in *B. fragilis* an another polysaccharide, PS-C was described [18]. The capsule of some *B. fragilis* strains is responsible for the abscess formation, which is the only anaerobic bacteria, that can induce abscess as solo bacterium [19]. Pili and fimbriae play role in the adhesion of *B. fragilis*; treatment with trypsin inhibit the haemagglutination and adhesion to human cells; while lektin-like adhesins reported to have got affinity to sialic rich macromolecules [19]. Fibrils are special structural elements, which are shorter than pili; about the fuction in adhesion is still little known so far [19]. *B. fragilis* can produce glycocalyx and involved in biofilm formation [19]. LPS in *B. fragilis* has got a significantly lower toxic activity than that of *E. coli* [4]. Outer membrane proteins (Omp), e.g. OmpA1 is an important in maintaining membrane structure of *B. fragilis*, and the membrane contains special iron-regulated hem uptaking proteins as well [20]. *B. thetaiotaomicron* with 163 homologues of SusC and SusD proteins is enable to attach to mucus glycans [9]. *B. fragilis* produce various enzymes: protesases are important in the digestion of food and additional enzymes are produced by certain members of this genus with histolytic activity (chondoitin sulfatase, hyaluronidase, lysozyme, lecithinase, deoxyribonuclease, phosphatase, and lipase) and fibrinolysin [4]. In some *B. fragilis* strains two hemolysins (HlyA and HlyB) were discovered [21]. Neuraminidase can cleave polysaccharides, remove terminal sialic acid from host cell surfaces and IgG [4]. The chemical structure of *B. fragilis* LPS is different form those extracted from most aerobic gram-negative bacteria and a very low toxic activity for mice after iv. injection was demonstrated [4]. Myers *et al.* proved in 1984 the patogenetic role of *B. fragilis* in diarrhoea [22]. Some *B. fragilis* isolates can express enterotoxin (BFT), known as enterotoxigenic *B. fragilis* (ETBF). The enterotoxin, known as fragilysin has a 20-kDa zinc-dependent metallo-protease chemical structure [23]. The toxin is encoded by *bft* gene, which is located in the 6 kb *B. fragilis* pathogenicity islet (BfPAI) [24], three allelic isoforms (*bft-1, -2* and *-3*) of this gene have been identified so far; *bft-3* allele was described in the Far East [23]. The fragilysin can cause diarrhoea in children aged mainly one to five years [23]. BFT is able to cleave E-cadherin, the intercellular adhesion protein forming the zonula adherens of intestinal epithelial cells, and cause reversible morphologic changes in colon carcinoma cell

line HT29/C1 [25]. Preliminary evidence suggests that enterotoxigenic *B. fragilis* may play role in the pathogenesis of inflammatory bowel diseases (IBD, e.g. ulcerative colitis, Crohn's disease), irritable bowel syndrome and colon cancer [26,27]. Some international research groups (e.g. Sears *et al.* [25], Ulger Toprak *et al.* [27], Choi *et al.* [28] etc.) investigated the *bft* gene; in 2006 in Hungary Nagy *et al.* published a minireview investigated the prevalence of *bft* gene and its isotypes of 275 intestinal and extraintestinal clinical samples [29]. Members of the cysteine protease families are widely distributed in prokaryotes and eukaryotes as well. Two cyteine protease types with pathogenetic role in *B. fragilis* have been recently described: *bfp1-4* genes encoding C10 and *fpn* gene encoding C11 proteases (fragipain). Recently it has been demonstrated that *B. fragilis* strains produce a special protease called fragipain (Fpn), which is a member of the C11 protease family (belonging to the CD clan of cysteine protease). Fragipain is required for the activation of fragilysin, and Choi *et al*. also hypothesised that activation of fragilysin may play a role in the progression of sepsis caused by ETBF srains [28]. The C10 protease is suspected of having a potential role in the pathogenesis in immune dysregulation, inflammatory bowel disease, irritable bowel syndrome. The fragipain activates *B. fragilis* enterotoxin and may play a role in the progression of sepsis [28,30]. *Bacteroides* species are able to multiply in the presence of oxygene in amount of nmol. In the genome of *B. fragilis* several reactive oxygene species (ROS) damaging enzyme genes (catalase: *kat*, superoxide dismutase: *sod*, alkyl-hydroperoxide reductase: *ahpC* etc.) [31]. *B. fragilis* has got the ability to evade host immune respone: the capsule protects the germ against phagocytosis and complement mediated killing [4]. These bacteria can decrease the nitric oxide (NO) production by inhibiting of inducible nitric oxide synthase enzyme (iNOS) of the macrophages, inhibiting thier response; however, Deng *et al.* described the *B. fragilis* strain ZY-312; which increased the nitric oxide production of macrophages *in vitro* [32].

I.4. ROLE OF *B. FRAGILIS* GROUP IN HUMAN INFECTIONS

The spectrum of anaerobic infections is quite wide and can be severe or even life-threatening. These infections are usually polymicrobial, caused by aerobic, facultative and obligate anaerobic bacteria and members of *B. fragilis* are found in most of these infections with an associated mortality of more than 19% [4]. The most severe infections caused by *B. fragilis* group isolates are intraabdominal abscesses, gangrenous appendicitis, gynecological, skin- and soft tissue infections, brain abscesses and sepsis [4]. Anaerobic bacteraemia accounts for 0.5–11.8% of all positive blood cultures [33], with very high mortality rate of (15–35%) [34], Brook reported that the most common clinically relevant isolates are strains belonging to

B. fragilis group (70%) [35]. The intraabdominal infections are caused by usually the rupture of diverticula, perforation of appendicitis, cancer, surgical wound or damage of gut wall. Chronic cholecystitis, visceral and intraabdominal abscesses and gangrenosus appendicitis are also polymicrobial by aerobic and anaerobic bacteria [35]. During the early phase of the infection, as bacterial synergism, the facultative anaerobic bacteria (e.g. *E. coli* or *Streptococcus sp.*) invade the intraabdominal cavity, and after the reduction of oxido-reduction potential *Bacteroides* species are able to grow [4]. These types of infections need surgical corrections or drainage and appropriate antimicrobial administration. From the cervical and vaginal flora *Bacteroides spp.* could be isolated in high numbers also. *Bacteroides spp*. and gram-positive anaerobic cocci (GPAC) were more commonly isolated from women's samples with cervicitis than those without cervicitis. In the vagina the carriage rate of *Bacteroides* species in pregnant and non-pregnant women is 0-6% [36]. *Bacteroides* species are usually isolated from ovaries, fallopian tubes and Bartholin's abscesses, *B. thetaiotaomicron* can be recovered from pelvic inflammatory disease [4]. The origins of the Central Nervous System (CNS) infections-including brain abscess, subdural or epidural empyema and meningitis caused by *Bacteroides*-are necrotizing enterocolitis, gastric perforation, aspiration pneumonitis, ventriculo-peritoneal or ventriculo-atrial shunt, lung abscess, pneumonitis, septicemia, chronic otitis, dental infections and less frequently sinusitis and mastoiditis [4,37]. Septic arthritis and osteomyelitis are rare, associated with hematogenous spread, prosthetic joint, rheumatoid arthritis or trauma [4]. The endocarditis and pericarditis caused by *B. fragilis* are very rare forms of infections [4,38]. Some authors implicated the pathogenetical role of the fragilysin in IBD; the pathogenesis of the IBD has been not clearly elucidated yet [39]. IBD is likely to be a multifactorial and heterogeneous disease, in which genetic and environmental factors play an important role [40]. Bamba *et al.* found that the serum antibody titer against the 26-kDa outer membrane protein of *B. vulgatus* is higher in patients with ulcerative colitis than in control group. This finding indicate that *B. vulgatus* with a 26-kDa protein outer membrane protein may play role in the pathogenesis of ulcerative colitis [41].

I.5. ANTI-ANAEROBIC ANTIBIOTICS AND RESISTANCE MECHANISMS

The number of the anti-anaerobic antibiotics is quite limited, which are the followings: some beta-lactams: cephamycins (e.g. cefoxitin), β-lactam/β-lactamase inhibitor combinations, carbapenems, 5-nitroimidazoles, clindamycin, macrolides, tertacycline, tigecycline, chloramphenicol and fluoroquinolones. According to the recent data among the anaerobic bacteria the *Bacteroides* strains are the most resistant against antibiotics [42,43]. The

background of the failure of the antimicrobial therapy are: insufficient number of cultures and susceptibility testing, mixed infection, not proper surgical technique, penetration of antibiotics, pharmacokinetics, natural and acquired resistance. The therapy of the undrainable abscesses is very difficult: fibrotic capsule inhibits the penetration of the antibiotics, presence of degradation enzyme, low pH, high osmolarity are the most important factors to inhibit the effect of the antibiotics [42].

I.5.1. Beta-lactams

The β-lactams are bactericidal antibiotics, that inhibits the cell wall synthesis. The resistance mechanisms against β-lactams among the *Bacteroides* isolates are: the production of *β*-lactamase enzymes, decreased permeability and expression of low affinity Penicilline-Binging Proteins (PBP). More than 90% of the *B. fragilis* group strains produce *β*-lactamase enzymes [44-46]. Two classification of *β*-lactamases are widespread: the Ambler classification based on molecular structure [44,45]; the Bush-Jacoby-Medeiros classification on the enzyme function [44]. The Ambler classification devides the *β*-lactamases into four classes: the members of class A contain serine in their active centre, class B enzymes are known as metallo-*β*-lactamases (MBL) containing Zn^{2+} ion, Class C *β*-lactamases are known as AmpC, Class D members are the OXA *β*-lactamases [44]. In the Bush-Jacoby-Medeiros classification differentiate the enzymes into three groups: Group 1 enzymes are the cephalosporinases, members of the Group 2 are the serine *β*-lactamases and Group 3 *β*-lactamases are known as the MBLs [45]. Rogers *et al.* described the *cepA* cephalosporinase gene (Group 2e, Class A) [46], the members of Group 2e are inducible, chromosomal enzymes; that are able to hydrolize penicillins and most of the cephalosporins (except cefoxitin) including the extended-spectrum cephalosporins and to be inhibited by *β*-lactamase inhibitors [46]. Among the *Bacteroides* strains another Group 2e resistance gene has been described, the *cfxA*, which is responsible for the cefoxitin resistance and was found on the mobilizable transposon Tn4555 and possesses the ability of spreading by conjugation among *Bacteroides* isolates [47]. The carbapenem resistance is associated with the chromosomal *cfiA* gene (Group 3, Class B), which encodes Zn^{2+}-dependent metallo-*β*-lactamase. For the expression of *cfiA* gene the presence of an IS element (e.g. IS*613*, IS*1169*, IS*614B*, IS*4351*, IS*1186* or IS*1187*) required in the upstream region [48]. *B. fragilis* Division I strains harbour *cepA* gene, but not *cfiA*; Division II isolates contain *cfiA* gene, but are *cepA*-negative [7,49]. As for the other resistance mechanisms, the production of *β*-lactamase is associated with decreased permeablility or the poor affinity to

PBPs. In *B. fragilis* three major PBPs (91 kDa, 80 kDa and 69 kDa) have been described so far, while two minor (63 kDa and 47 kDa) have been detected as well [50].

I.5.2. Fluoroquinolones

The broad spectrum, bactericidal fluoroquinolones are the fluorized derivates of the nalidixic acid; which target points are the DNA gyrase and topoisomerase IV enzymes, inhibiting the bacterial DNA replication [4]. Both enzymes are tetramers consisting of two subunits (DNA gyrase: GyrA and GyrB; topoisomerase IV: parC and parE), which are encoded by *gyrA, gyrB, parC* and *parE* genes [51]. The one widespread fluoroquinolone resistance mechanism is the changing of the target point, the substitution in the Quinolone Resistance-Determining Region (QRDR) of mainly in GyrA and GyrB enzymes (ParC and ParE substitution can be detected usually in gram-positive bacteria), so that the fluoroquinolones can much with lower affinity bind to these enzymes. The most common substitutions in *Bacteroides* isolates are: Ser82Phe, Ser82Leu, Asp81Asn etc. (GyrA); Leu415Val (GyrB) [51]. The other important mechanism is the overexpression of active multidrug efflux genes; two major MDR families have been discovered in *Bacteroides* genus so far: the Resistance-Nodulation-Division (RND) superfamily (encoded by *bmeB1-16* genes) and the Multidrug and Toxic Compound Extrusion (MATE) family (encoded by *bexA* gene) [52].

I.5.3. Macrolide-Lincosamide-Streptogramin B (MLS_B)

Members of the Macrolide-Lincosamide-Streptogramin B group drugs are bacteriostatic, structurally diverse, but the the mechanism of action is common. They bind to the 50S ribosomal subunit to inhibit the proteine synthesis by altering the binding of the peptidyl-tRNA and inhibition of the movement of the peptide chain [53]. Three resistance mechanisms have been discovered so far: the first is the methylation of a single adenine in the 23S rRNA by rRNA methylase enzymes (encoding by *ermB, ermF, ermG, ermFU* genes), which leads reduced binding of drug to the ribosome [51]. Efflux pump mechanism can be observed, mediated by *msrSA* and *mefA* genes; *msrSA* gene encodes an active erythromycin-efflux pump [47]. The resistance genes can be found on transferable plasmids (pBF4, pBFTM10 (pCP1) and pB1136), while the *ermF* gene on transposons (Tn4351 /pBF4/, Tn4400 /pBFTM10/ and Tn4551 /pB1136/) and *ermFU* on conjugative transposon. The third mechanism is the enzymatic modification by an O-nucleotidyl-transferase encoded by the *linA* gene [47].

I.5.4. Tetracycline and tigecycline

The tetracycline derivates are broad spectrum, bacteriostatic antibiotics, that bind reversible to the 30S ribosomal subunit preventing the linkage of aminoacyl-tRNA to the ribosome, inhibiting the proteine synthesis [53]. The first resistance mechanism is the active efflux, encoded by the *tetA, tetB, tetC, tetD, tetE* etc. genes; the efflux proteins belong to the Major Facilitator Superfamily (MFS). These efflux pumps do not confer resistance to the newly developed glycylcycline derivate, the tigecycline, which is a broad spectrum drug and the resistance mechanism(s) among anerobic bacteria have not been elucidated fully yet [53]. Many *Bacteroides* strains are able to produce ribosomal protection proteins, among their genes the most important are *tetQ, tet32, tet36, tetM, tetO* etc. The enzymatic modification of tetracycline (encoding by *tetX* and *tet37* genes) is not important among anaerobic bacteria, because the 44-kDa protein encoded by *tetX* requires both oxygene and NADPH for its activity [53]. CTnDOT carries often tetracycline (*tetQ*) and MLS_B resistance genes (*ermF*) simultaneously [54].

I.5.5. 5-nitroimidazoles

The 5-nitroimidazoles (metronidazole, tinidazole) are bactericid prodrugs, only after enzymatic activation by the pyruvate:ferredoxine-oxydoreductase (PFOR) enzyme can produce nitroamine radicals that damage DNA at very low redox potential in oxygene free environment. In anaerobic bacteria the ferredoxin-like Fe-S proteins play important role in the maintanace of low redoxpotential. The mechanism of action of metronidazole has not been clearly and completely clarified; and the resistance mechanism to metronidazole is also complex. Some *Bacteroides* isolates express 5-nitroimidazole reductase enzymes (encoded by *nimA-J* genes), that converts the nitro-group into the non-toxic amino-imidazole, activated by IS-elements (e.g. IS*1168*, IS*1170*) [55]. The other mechanisms are the down-regulation of the PFOR enzyme; and the upregulation of lactate-dehydrogense enzyme, through the lack of substrate via the biochemical conversation of the pyruvate into lactate, the activity of the PFOR enzyme decreases. Other mechanisms may play role in the 5-nitroimidazole resistance: multidrug efflux pumps (RND family), increased DNA repair, mutation of the *feoAB* iron transport system [55].

I.5.6. Cloramphenicol

Chloramphenicol is a broad spectum, bacteriostatic antibiotic drug that inhibits the protein synthesis by binding to the peptidyl-transferase at the ribosomal 50S subunit [55]. The first and most frequent mechanism of resistance to chloramphenicol is the enzymatic

inactivation by acetylation encoded by *cat* gene; however, efflux systems, target site modification, permeability decreasing and inactivation by phosphotransferase mechanisms were described [56]. The resistance to this drug among *Bacteroides* strains is very rare [55]. Chloramphenicol was regarded to be a very effective drug in serious anaerobic infections, especially in the infections of the CNS, because of the excellent penetration ability of the blood-brain barrier; however, this drug might cause severe side effects, e.g. fatal aplastic anemia, "gray baby syndrome" in neonates, optic neuritis and reversible, dosage-dependent leukopenia [57].

I.6. GENETIC ELEMENTS RESPONSIBLE FOR THE EXPRESSION AND SPREADING OF ANTIBIOTIC RESISTANCE GENES

I.6.1. Insertion sequences (IS elements, ISs)

The IS elements are defined as a small (<2.5 kb) cryptic segments of DNA with the capability of insertion at multiple sites [58]. These elements encoding transposase enzymes, serve to promote plasmid excision and integration; and many ISs have been shown to be a promoter to activate the expression of the neighbouring genes [58]. The most important ISs of *Bacteroides* genus are IS*942*, IS*1224*, IS*1168*, IS*1169*, IS*1170*, IS*1186*, IS*4351* and IS*4400*. The IS-born promoters were discovered in the upstream region of carbapenem (*cfiA*), macrolid (*erm*) and metronidazole resistance genes (*nimA-J*) [58-60]. The ISs can be part of transposons or independent from them, the most common *Bacteroides* transposons are Tn4351, Tn4451 and Tn4400 [58]. The IS*942* is positioned in the upstream region of *cfiA* gene, this element is 1598 bp and possesses an open reading frame (ORF) [58]. The IS*1170* is closely related to IS*1169* and 70% identical to IS*942*, found on 5-nitroimidazole resistance (Nm^r) plasmid pIP417, inserted into the upstream region of *nimC* gene. The IS*1186* is very similar to IS*1168* and originally positioned in the upstream region of *nimA* gene [60], while the IS*1169* was found near *nimD* gene [58]. The *cfiA*-positive *B. fragilis* strains harbour at least one of the three IS elements, 68% of the strains contain IS*4351*, 43% IS*1186* and 18% IS*942*; the common prevalence of IS*4351* and IS*1186* was 37% [58].

I.6.2. Conjugative transposons (CTns)

The conjugative transposons (CTn) are special genetic elements in both gram-negative and gram–positive bacteria that are integrated into a chromosome or plasmid. They can excise and encoding conjugative apparatus (CA), they can transfer to a recipient germ by conjugation

[61]. The conjugative transposons, sized 52-150 kb are highly distributed among *Bacteroides* isolates, more than 80% of them harbour at least one CTn [62]. During their transfer they produce a covalently closed circular intermediate containing *oriT* sequence to allow conjugation [63]. CTn-s are able to transfer other genetic elements, such as plasmids and transposons (Tn4399 or cefoxitin resistance gene *cfxA* gene carrier Tn4555) [58]; they can mediate the excision and mobilization of Nonreplicating *Bacteroides* Units (NBUs) as well. The most important CTns of *Bacteroides* isolates are BFT-37, CTn86, CTn9343 (*B. fragilis*); CTnDOT, CTnERL, TcrEmrDOT, CTnGERM1 (*B. thetaiotaomicron*) and CTn341 (*B. vulgatus*) etc. [58,64-67]. Many *Bacteroides* isolates harbour a family of CTns, which is responsible for tetracycline resistance, and their members are known as tetracycline resistance elements, Tc^r-s [61]. The tetracycline resistance gene, *tetQ*, and two other genes (*rteA*, *rteB*) of the regulatory system *rteABC* cluster constitute the *tetQ-rteA-rteB* operon. Following the tetracycline stimulation, the RteA protein activates RteB, which enhances the expression of the *rteC* gene, which is also an important component of the *tetQ* gene expression [61]. Large number of *B. fragilis* group strains carry CTns containing Macrolide-Lincosamide-Streptogramin B (MLS_B) resistance genes, known as erythromycin resistance (Em^r) genes as well, e.g. *ermB* (CTnBST), *ermF* (CTnDOT) and *ermG* (CTnGERM1) [68,69]. Many of the CTns, e.g. CTnDOT of *B. thetaiotaomicron* harbours *tetQ* and one of the *erm* genes, e.g. *ermF* simultaneously [70].

I.6.3. Mobilizable transposons (MTns)

Mobilizable transposons (MTns) can transfer from the donor to the recipient only with "helper elements", such as conjugative transposons or plasmids into philogenetically different species as well [61]. The MTns located on chromosome, have got smaller size (5-12 kb) than CTns and harbour genes for the excision and integration of genetic elements [61]. Among the *Bacteroides* species several transposons have been described and characterized so far, e.g. Tn4399, Tn5520, Tn4351, Tn4400, NBU3 (*B. fragilis*), Tn4555 (*B. vulgatus*), NBU1, NBU2 (*B. uniformis*) [58]. The Tn4351 and Tn4551 are flanked by IS*4351*; the Tn4400, which is very similar to Tn4351, is flanked by IS*4400*; these transposons mediate the MLS_B resistance genes (*ermF* or *ermFS*) expression. The 10-12 kb NBU1 and NB2 were firstly described in *B. uniformis* strain 0061, that are small, plasmid-like elements. The NBUs (NBU1, NBU2 and NBU3) were found only in the presence of Tc^r element and the excision and the transfer by conjugation requires Tc^r Em^r DOT-type elements. In absence of tetracycline the NBUs are

integrated into the chromosome [58]. The excision and integration of NBUs are very similar to λ phages, and the NBU integrase gene (*int*) is a member of λ integrase family [58]. The NBUs contain a mobilization gene (*mob*), which encodes the Mob protein with *oriT* binding and nicking activity [71].

I.6.4. Plasmids

Bacteroides strains can harbour two different types of plasmids: cryptic and antibiotic resistance plasmids. Two groups of the resistance plasmids have been discovered so far: conjugative and mobilizable plasmids. These plasmids can replicate independently, may integrate into chromosome and *oriT* sequence and *trans*-acting mobilization gene(s), which allows the transfer by conjugation. Approximately 20-50% of these isolates contain cryptic plasmids sized 2.7 to >80 kb, which can replicate extrachromosomally and integrate into chromosome as well [58]. Some studies reported three major classes of these plasmids, which are: Class I (2.8-kb), Class II (4.2-, 5.0- and 7.9-kb) and Class III (5.5-kb) [70]; and Sóki *et al.* described three more classes: class I (1.8 MDa), class II (2.6 MDa) and class III (3.7 MDa) [71]. Despite the widespread distribution of cryptic plasmids they do not harbour any resistance genes [72,73]. In the *Bacteroides* strains some antibiotic resistance plasmids have been described, e.g. pBII36 (80 kb), pBFTM10 (15 kb) and pBF4 (41 kb), which plasmids harbour MLS_B resistance genes (*erm*). The MLS_B genes are located on transposons Tn4351, Tn4400 and Tn4551 [58]. The 5-nitroimidazole resistance plasmids (Nm^r) sized 7 to 56 kb are pIP417, pIP419 and pIP421 have been described so far, which harbour *nimA*, *nimC* and *nimD* genes [58]. In *B. uniformis* the plasmid-linked chloramphenicol acetyltransferase gene (*cat*) was described [74], the carbapenem resistance gene (*cfiA*) is positioned also on a 6.4-kb plasmid [75].

I.7. IMPORTANCE OF ANTIBIOTIC SUSCEPTIBILITY TESTING AND THE SURVEILLANCE

The routine antibiotic susceptibility testing among anaerobic bacteria in all clinical situations is not recommended by the Clinical and Laboratory Standards Institute (CLSI), only is special indications [76]. Antibiotic susceptibility test is performed (i) in case of serious infection, (ii) the sample was taken from sterile body site, (iii) infection failed to response to empirical therapy, (iv) relapse after initially successful therapy, (v) few susceptibility data available, (vi) when isolate is often resistant, (vii) when prolonged therapy required [75].

Susceptibility testing is recommended in epidemiological surveillance and in case of highly virulence bacteria, such as *Bacteroides, Prevotella, Fusobacterium, Clostridium spp., Bilophila wadsworthia* and *Sutterella wadsworthensis* [75]. The tested antibiotics include penicillin, β-lactam/β-lactamase inhibitor combinations, clindamycin, metronidazole and a carbapenem, if needed, cefoxitin, tigecycline and moxifloxacin can be tested. Only a few data are available on the resistance trends of anaerobic bacteria (hospital–level, regional-national, surveillance studies) is restricted to the studies published by anaerobic reference laboratories or some national collaborations, in comparison to the aerobic bacteria. Although the variations between different geographical regions are notable, common tendencies can be observed. While three decades ago the antibiotic susceptibility pattern of anaerobic bacteria was straightforward, nowadays we cannot so easily predict the efficiency of the chosen empirical therapy. Clinicians can no longer "expect" certain drugs to work in anaerobic infections because they showed potent activity before. With the use of broad-spectrum antimicrobials in addition to the suitable surgical measures, the issue of emerging resistance of anaerobic bacteria was maybe less obvious. But if we observe the data from the (inter)national surveillance reports from both the United States and Europe, the same trends can be observed: steadily growing resistance to some of them rendered completely useless. In Hungary only a very few studies have been performed so far investigated the antibiotic susceptibility pattern of *B. fragilis* group isolates [77,78]. Our study was the first comprehensive antibiotic susceptibility study, coordinated by one clinical microbiological centre performed with uniform criteria (e.g. collection of the first 100 non-repeating isolates from each centres, agardilution method) and with so high number of isolates. The comparison of our data and the previous Hungarian studies is quite hard, because of the difference of breakpoints and the antibiotic susceptibility methods. Some publications report the emergence of MDR resistant isolates (especially within the *B. fragilis* isolates), harbouring multiple resistance genes or with a combination of intrinsic and acquired resistance mechanisms [79-86]. In these cases, the bacteria were usually termed MDR if they were resistant to three-four antibiotic classes besides metronidazole (due to *nim* 5-nitroimidazole resistance genes) and the carbapenems (a metallo-*β*-lactamase encoded by *cfiA* or *ccrA* genes) [79-86]. The significance of the above mentioned tendencies in further underlined by the fact, that treatment failure has been described in empirical treatment in cases of anaerobic bacteraemia, as result of a MDR *B. fragilis* infection. What makes this problem even more insidious is the fact that the correlation between the presence of a MDR anaerobic strain and clinical failure is hard to prove. As the consequence of the extended usage of antibiotics, not only among aerobic but also the

anaerobic bacteria appeared the MDR isolates; however, MDR *Bacteroides* isolates have rarely been published so far [87]. 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 the expression. The range of the bacterial genome size is between 0.46 and 9.7 Mb [88], and the *Bacteroides* isolates can be the reservoir of different antibiotic resistance genes, which can get passed by horizontal gene transfer (HGT) [89]. Besides the antibiotic resistance genes (*cfiA, cfxA, cepA, nim, tetQ, erm* etc.) the overexpression of the genes of the RND or MATE efflux pumps systems (*bmeB1-16, bexA*) play important role in the MDR isolates [4]. Mobile genetic elements (plasmids containing resistance determinants, insertion sequence (IS) elements, transposons) have a significant role in the spread of the MDR phenotype in anaerobes.

II. AIMS OF THE STUDY

The international studies reported increasing antimicrobial resistance among *B. fragilis* group isolates in case of some antibiotics. In Hungary, during the last two decades no comprehensive antimicrobial susceptibility survey 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; and during the last one and half decades the number of *B. fragilis* isolates in our Institute has increased almost threefold. 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.

Our aims were the followings:

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

2. Epidemiological investigation of antimicrobial susceptibility of 400 clinical *B. fragilis* isolates from different geographical area in Hungary and compare the resistance trends 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 Hungarian *B. fragilis* group strains.

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 FOR THE DENTIFICATION OF *B. FRAGILIS* GROUP ISOLATES

III.1.1. Bacterial strains

A total of 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. 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. Local laboratories cultured and identified the examined strains according to standard laboratory procedures for anaerobic bacteria. The first identification was performed by MALDI-TOF MS (Bruker Daltonik, Germany) all of the participating clinical microbiological centers. Species were distributed as follows during the first routine identification. Before the final MALDI-TOF MS analysis in Szeged, all the examined 400 strains were cultured on Schaedler agar (bioMérieux, France) for 48 hours, at 37 °C in anaerobic chamber (Perkin Elmer, UK) under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2).

III.1.2. MALDI-TOF MS

The strains were identified in each center and also in Szeged by MALDI-TOF MS (Bruker Daltonik, Germany) with Biotyper Version 3.0 software containing 5989 mass spectra of reference strains of aerobic, anaerobic bacteria and fungi. The re-identification of each strain in Szeged was performed with three parallel measurements and the same conditions (strains and chemicals). Results with the best log score values were accepted. The measurement mode was microflex, the parameters were: linear positive ion mode with a laser frequency of 20 Hz, LT: ISI 20 kV, IS2 18.5 kV, lens 8.5 kV, PIE 250 ns, no gating, range: 20-20 000 Da [90]. A small amount of one colony was spotted on the target plate, 1 µl of 70% aqueous formic acid and after drying 1 µl of MALDI matrix (α-cyano-4-hydroxycinannamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid) were added to the spot. Interpretation of log score values were as follows: 0.000-1.699: unreliable identification; 1.700-1.999: genus level identification; ≥2.000: species level identification. The *B. fragilis* strains were categorized as genetic Division

I and Division II by MALDI-TOF MS, as described by Fenyvesi *et al.* earlier [91]. We applied *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29742 strains as controls.

III.1.3. Rapid ID 32A

A sum of 21 strains with contradictory results of identification and re-identification obtained by MALDI-TOF MS were checked by traditional biochemical test kit of rapid ID 32A (bioMérieux, France) method according to the manufacturer's instructions. Suspension with 4 McFarland turbidity was prepared from 48 h subculture in 2 ml of sterile suspension medium and dropped 55 µl into each cupule. The cupule for urease enzyme was overlaid with mineral oil. After covering the strips, they were incubated under aerobic conditions for 4 h at 37 °C. To nitrate and indole cupules the appropriate reagents were added and these tests were read after 5 min. Catalase production was also investigated directly with 15% hydrogene-peroxide. The biochemical profile was analyzed by computer with a specific database (analytic profile index, version 3.2) provided by the manufacturer. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29742 were used as control strains, the excellent identification level was set to 95.0%.

III.1.4. RT-PCR

These 21 strains with contradictory results were identified by 16S rRNA gene sequencing as well. DNA templates for PCR analyses were prepared as follows: one colony of each isolate was suspended in 100 µl of distilled water and heated at 99.5 °C for 12 minutes in a dry bath. The RT-PCR reactions for amplification of 16S rRNA gene was performed using 30 µl total volumes, containing 15 µl 2x SYBR Green qPCR Master Mix (BioTool, USA), 10.2 µl water, 0.6 µl of E8F (5'-AGAGTTTGATCCTGGCTCAG-3') and E533R (5'-TIACCGIIICTICTGGCAC-3') primers (concentrations: 35-35 pmol/µl), 0.6 µl ROX (BioTool Swiss AG, Switzerland) and 3 µl of DNA templates. StepOne RT-PCR machine (Applied Biosystems, USA) was used for the PCR cycling and detection: 95 °C 10 min, followed by 35 cycles of 95 °C 15 sec, 56 °C 20 sec, 72 °C 30 sec and one cycle of 72 °C 75 sec and a melting curve detection from 72 °C to 95 °C.

III.1.5. 16S rRNA gene sequencing

The DNA amplicons from RT-PCR reactions (proportional scale-up to 30 µl) were purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan). The templates were sequenced with ABI BigDye® Terminator Version 3.1 kit in Series Genome Analyzer 3500 (Life Technologies, USA). The obtained sequencing data were analyzed by

NCBI BLAST (http://www.ncbi.nim.nih.gov/blast/cgi) and leBiBi software (http://pbil.univ-lyon1.fr/bibi), the reliable identification level was set to 98.0%.

III.2. ANTIMICROBIAL SUSCEPTIBILITY TESTING

III.2.1. Agar dilution method

The Minimal Inhibitory Concentration (MIC) values for ten antibiotics were determined with agar dilution method according to the recommendation of the CLSI [76]. The antibiotics tested included ampicillin, cefoxitin, tetracycline, tigecycline, chloramphenicol (Sigma-Aldrich Chemie GmbH, Germany), amoxicillin/clavulanic acid (GlaxoSmithKline, UK), meropenem, moxifloxacin (Fresenius Kabi, Germany), clindamycin (Pfizer, USA), metronidazole (TEVA, Israel). The ranges applied for the antibiotics were the following: ampicillin (2-256 mg/l), amoxicillin/calvulanic acid (0.064/0.032-16/8 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). For the interpretation of the MIC-value, if it is available in guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (ampicillin, amoxicillin/clavulanic acid, meropenem, clindamycin, metronidazole), if there is no EUCAST breakpoint, CLSI guidelines (cefoxitin, moxifloxacin, tetracycline and chloramphenicol) were used (Table 2) [76,92]. As the tigecycline breakpoints among *Bacteroides* species have not yet been established either by EUCAST or CLSI, the breakpoints published by Nagy *et al.* were applied for the interpretation [78]. Here, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 served as control strains.

Table 2.: EUCAST and CLSI breakpoints for the interpretation of MIC values of *B. fragilis* group isolates

Antimicrobial agents	EUCAST [92]		CLSI [76]		
	S	R	S	I	R
Ampicillin	**≤0.5**	**>2**	**≤0.5**	**1**	**≥2**
Amoxicillin/clavulanic acid	**≤4**	**>8**	**≤4/2**	**8/4**	**≥16/8**
Cefoxitin	**ND**	**ND**	**≤16**	**32**	**≥64**
Meropenem	**≤2**	**>8**	**≤4**	**8**	**≥16**
Clindamycin	**≤4**	**>4**	**≤2**	**4**	**≥8**
Metronidazole	**≤4**	**>4**	**≤8**	**16**	**≥32**
Moxifloxacin	**ND**	**ND**	**≤2**	**4**	**≥8**
Tetracycline	**ND**	**ND**	**≤4**	**8**	**≥16**
Tigecycline	**ND**	**ND**	**ND***	**ND***	**ND***
Chloramphenicol	**ND**	**ND**	**≤8**	**16**	**≥32**

ND: No data, S: Susceptible, I: intermediate, R: resistant

*ND: susceptible: ≥4; intermediate: 8; resistant: >16 [78]

III.2.2. Real-Time-PCR

The presence of the *cfiA* gene in *B. fragilis* strains belonging to Division II was confirmed by RT-PCR, as described by Eitel *et al.* [47]. To get DNA template, one colony of each strains was suspended in 100 µl of distilled water and heated at 99.5 °C for 12 minutes in a dry bath. Primer sequences applied for *cfiA* RT-PCR were: forward: AATCGAAGGATGGGGTATGG and reverse: CGGTCAGTGAATCGGTGAAT, PCR conditions were: 94 ºC 15 s, 59 ºC 1 min, 72 ºC 30 s, 40 cycles. The total volume of 10 µl of PCR-reaction mixture was composed of 5 µl 2x PCR "master-mix" (iQ, Bio-Rad, USA), 0.7 µl (35-35 pmol) of each primer, 1 µl template DNA, 0.5 µl EvaGreen (Biotium, USA) DNA-binding fluorescent dye (for the iQ "master-mix") dye. The amplification was performed in StepOne (Life-Technologies, USA) Real-Time PCR instrument [47].

III.2.3. Statistical analysis

The data values were analysed by using Fischer's Exact and Spearman correlation tests in the SigmaPlot 12 program in order to look for differences contained, and the significance

level was set to 0.05 (i.e. $p<0.05$). The antibiotic resistance data values were analysed via the chi-square test (χ^2-test) contained in SigmaPlot 12.

III.3. MOLECULAR INVESTIGATION OF MULTIDRUG RESISTANT STRAINS

III.3.1. Real-Time PCR

Among the 400 *B. fragilis* group isolates we found six MDR isolates. Molecular investigations of these strains were carried out in order to detect the 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 summarized in Table 3. Positive controls 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*) [47].

Table 3.: PCR reaction conditions of the investigated antibiotic resistance genes and genetic elements

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	

III.3.2. 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, USA).

III.4. *B. FRAGILIS* ENTEROTOXIN GENE AND ITS ISOTYPES AND THE C10 AND C11 CYSTEINE PROTEASE GENES

III.4.1. PCR detection of *cfiA, bft, bfp1-4,* and *fpn* genes

The presence of the *cfiA* gene in *B. fragilis* strains belonging to Division II was confirmed by RT-PCR, as described by Eitel *et al.* [47]. Using the procedure outlined by Sóki *et al.*, RT-PCR was performed for the detection of *bft* gene in the case of all 200 *B. fragilis*, using bftF and bftR primers [86]. For the typing of the *bft* gene, an internal fragment of *bft* gene was amplified with BTT1 and BTT2 primers and a melting point analysis was performed. These PCR products were purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan) and investigated with RFLP as well to differentiate the isotypes of the gene [87]. During the RFLP analysis according to the international literature, the following positive controls were used: *B. fragilis* R19811 (*bft-1*), *B. fragilis* ATCC 43858 *(bft-2)*, and *B. fragilis* GAI 96462 (*bft-3*). The expected lengths of the restricted fragments are 839 and 310 bp for *bft-1*; 575, 453 and 111 bp for *bft-2*; 839, 189 and 111 bp for *bft-3* [88,30]. An internal fragment of three *bft-1* and three *bft-2* harbouring *B. fragilis* isolates was sequenced with the ABI BigDye® Terminator Version 3.1 kit in the Series Genome Analyser 3500 (Life Technologies, USA) 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 in 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 using the following control strains: *B. fragilis* 638R (*bfp1-4)* and *B. fragilis* ATCC43859 (*fpn*) [28,88,30]. All of the RT-PCR tests were carried out using the StepOne RT-PCR machine (Applied Biosystems, USA). The PCR set-up for the detection of *cfiA, bft, bfp1-4* and *fpn* genes are summarized in Table 4.

Table 4.: PCR conditions of *cfiA, bft, fpn* and *bfp1-4* genes

Gene	Primer	Primer sequence (5' → 3')	PCR conditions
cfiA	cfiA-F	AATCGAAGGATGGGGTATGG	95°C 10 min; 35 cycles 95°C for 15 sec; 59 °C for 30 sec; 72 °C for 75 sec; melting 72-95 C°
	cfiA-R	CGGTCAGTGAATCGGTGAAT	
bft (RT-PCR)	bftF	CGAACTCGGTTTATGCAGTT	95°C 5 min; 35 cycles 95°C for 15 sec; 56 °C for 1 min; 72 °C for 30 sec; melting 72-95 C°
	bftR	GGATACATCAGCTGGGTTGT	
bft (RFLP)	BTT1	CATGTTCTAATGAAGCTGATTC	95°C 5 min; 35 cycles 95°C for 15 sec; 56 °C for 1 min; 72 °C for 30 sec; melting 72-95 C°
	BTT2	ATCGCCATCTGCTGTTTCCC	
fpn	C11_protease_F	ATTCGGCCGATGCAAATGTG	94°C 5 min; 30 cycles 94°C for 1 min; 54 °C for 1 min; 72 °C for 1 min
	C11_protease_R	CGGAATCTCGGTAGGGAAC	
bfp1	C10_protease_F1	GCGGTGAACAAAGAACGACA	95°C 10 min; 35 cycles 95°C for 15 sec; 59 °C for 30 sec; 72 °C for 75 sec; melting 72-95 C°
	C10_protease_R1	TCGCCTGAGCAACTGCAATA	
bfp2	C10_protease_F2	CGTACCAATTGCAATTGCGC	
	C10_protease_R2	AGCTCCCGTGGCTTTATCTT	
bfp3	C10_protease_F3	TTTGGAGTAGCAGCAGCAGA	
	C10_protease_R3	TTTCTGGTTTCGGGTGTTTC	
bfp4	C10_protease_F4	TACAACGGTGTTGGTGCAAG	
	C10_protease_R4	ACACAAATGCGCCACTTCAT	

III.4.3. Statistical analysis

The data were analysed by Fischer's Exact and Spearman correlation tests in order to look for differences using SigmaPlot 12, and the significance level was set to 0.05 (i.e. $p<0.05$).

IV. RESULTS

IV.1. VALIDATION OF MALDI-TOF MS METHOD FOR THE DENTIFICATION OF DIFFERENT *B. FRAGILIS* GROUP CLINICAL ISOLATES

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) and the best log score values were chosen for analysis. Among the results of three parallel MALDI-TOF MS re-identification, the best log score from the identification results was chosen. MALDI-TOF MS re-identification results of 21 strains with log score value range of 1.855–2.458 were confirmed by 16S rRNA gene sequencing method and rapid ID 32A. These isolates with discrepancy according to the re-identification in Szeged are four *B. fragilis* and 17 non-fragilis *Bacteroides* strains (seven *B. thetaiotaomicron*, one *P. distasonis*, one *B. cellulosilyticus*, one *B. salyersiae*, one *B. stercoris*, one *B. intestinalis*, one *B. vulgatus*, two *B. ovatus* and two *B. nordii*). The log score value of five strains among the examined 21 isolates with contradictory results was under 2.000 (1.993–1.802). 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) (Table 5). The database of rapid ID 32A does not contain the biochemical profiles of *B. cellulosilyticus*, *B. nordii*, *B. salyersiae*, and *B. xylanisolvens*, for this reason, four strains were not acceptable, whereas *P. distasonis* strains SY2 were identified as *Capnocytophaga sp.* In comparison of identification results with MALDI-TOF MS and rapid ID 32A, we reported only five concordant results (23.81%, 5/21). The quality of sequencing results was ≥98.0%, with the exception of strain SY9, which was identified with the level of 95.0%. In case of *Bacteroides* strains SY9, SY64, and SY81, the MALDI-TOF MS and 16S rRNA gene sequencing results were different (SY9: *B. intestinalis/B. cellulosilyticus*; SY64: *B. nordii/B. salyeriae*; and SY81: *B. ovatus/B. xylanisolvens*), because these strains are phylogenetically closely related and the protein patterns of these species are so similar that makes identification by mass spectrometry difficult. We accepted the sequencing results of *B. fragilis* SY23, *B. thetaiotaomicron* SY53 and *B. fragilis* SE33 strains. In the case of five isolates, the log score value of MALDI-TOF MS was bit lower than 2.000 (D2: 1.871; D4: 1.993; D71: 1.855; SY9: 1.916; and SY64: 1.802).

Table 5.: Comparison of results of three different identification methods of 21 *Bacteroides* strains

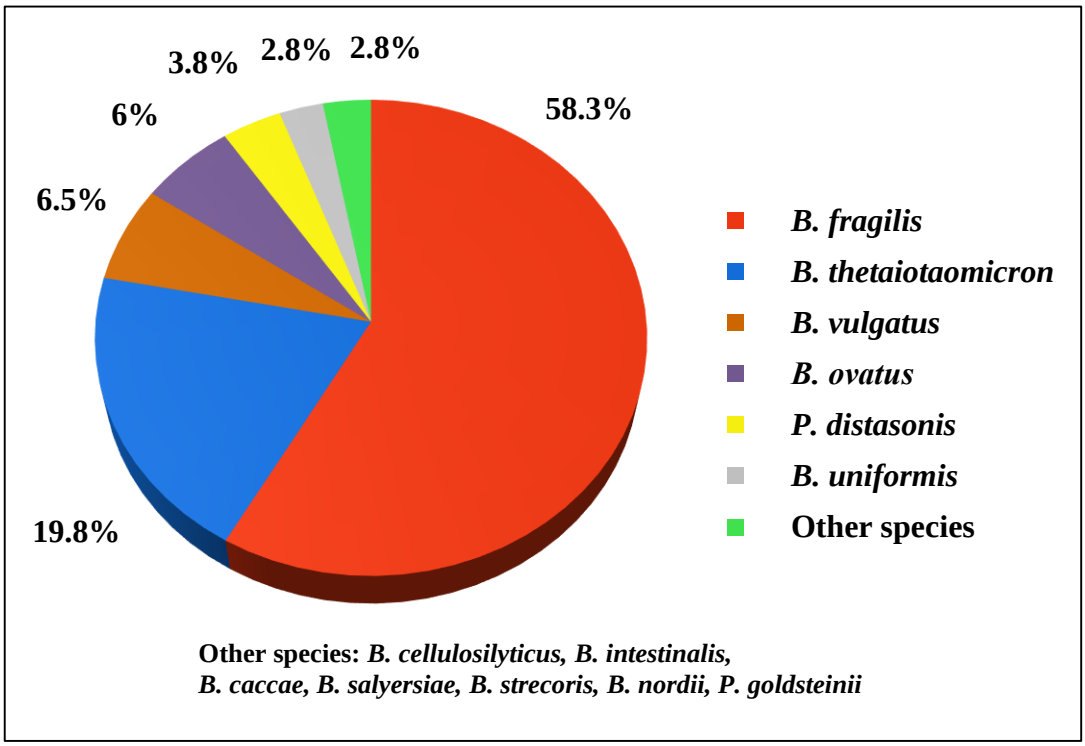
Strains	First identification with MALDI-TOF	Re-identification in Szeged with MALDI-TOF	Log score	Results of 16S rDNA sequencing (BLAST)	Quality of sequencing	Results of rapid ID 32A identification	Quality of identification
D2	*B. ovatus*	*B. thetaiotaomicron*	1.871	*B. thetaiotaomicron*	99%	*B. thetaiotaomicron*	99.8%
D4	*B. fragilis*	*B. thetaiotaomicron*	1,993	*B. thetaiotaomicron*	99%	*B. thetaiotaomicron*	99.7%
D39	*B. salyersiae*	*B. fragilis*	2,237	*B. fragilis*	95%	*B. uniformis*	39.6%
D46	*B. vulgatus*	*B. thetaiotaomicron*	2,188	*B. thetaiotaomicron*	99%	*B. thetaiotaomicron*	Very good quality
D63	*B. ovatus*	*B. fragilis*	2,458	*B. fragilis*	99%	*B. fragilis*	97.1%
D69	*B. uniformis*	*B. thetaiotaomicron*	2,129	*B. thetaiotaomicron*	99%	*B. caccae*	52.4%
D71	*B. uniformis*	*B. thetaiotaomicron*	1,855	*B. thetaiotaomicron*	99%	*B. uniformis*	Not accetable profil
SY2	*B. fragilis*	*P. distasonis*	2,359	*P. distasonis*	99%	*Capnocytophaga sp.*	Not acceptable profil
SY9	*B. vulgatus*	*B. intestinalis*	1,916	*B. cellulosilyticus*	95%	*P. distasonis*	82%
SY23	*B. fragilis*	*B. vulgatus*	2,072	*B. fragilis*	99%	*B. uniformis*	Not acceptable profil
SY25	*B. thetaiotaomicron*	*B. fragilis*	2,382	*B. fragilis*	99%	*B. thetaiotaomicron*	Not acceptable profil
SY53	*B. vulgatus*	*B. ovatus*	2,072	*B. thetaiotaomicron*	99%	*B. uniformis*	61.7%
SY64	*B. ovatus*	*B. nordii*	1,802	*B. salyersiae*	99%	*B. ovatus*	84.5%
SY77	*B. ovatus*	*B. fragilis*	2,081	*B. fragilis*	99%	*B. fragilis*	97.8%
SY81	*B. fragilis*	*B. ovatus*	2,232	*B. xylanisolvens*	99%	*B. caccae*	52.8%
SE33	*B. fragilis*	*B. thetaiotaomicron*	2.39	*B. fragilis*	99%	*B. fragilis*	96.5%
SE56	*B. thetaiotaomicron*	*B. stercoris*	2.357	*B. stercoris*	99%	*B. fragilis*	97.2%
SE57	*B. caccae*	*B. salyersiae*	2.008	*B. salyersiae*	99%	*B. caccae*	54.3%
SE59	*B. fragilis*	*B. thetaiotaomicron*	2.135	*B. thetaiotaomicron*	99%	*B. fragilis*	96.6%
SE67	*B. fragilis*	*B. cellulosilyticus*	2.327	*B. cellulosilyticus*	99%	*B. fragilis*	97.7%
SZ80	*B. ovatus*	*B. nordii*	2.043	*B. nordii*	98%	*B. ovatus*	43.3%

IV.2. INVESTIGATION OF ANTIBIOTIC SUSCEPTIBILITY

IV.2.1. *B. fragilis* group clinical isolates

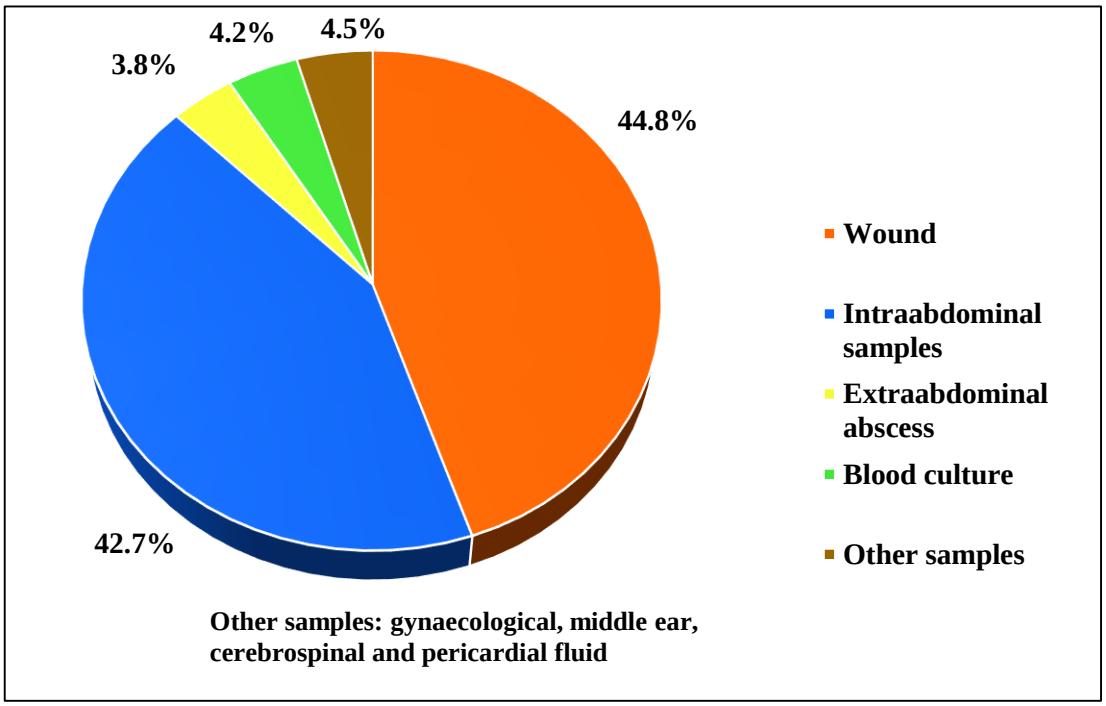
A total of 400 *Bacteroides* clinical strains were investigated, in case of 397 patients the samples were taken after 48 hours of admission to hospital; and there were only three patients, whose samples were taken by a General Practitioner (GP). Here, only 13.0% of the isolates were isolated from a pure culture and 87.0% from a mixed culture. 43.5% of the patients were female, 56.5% were male and they had an average age of 59.3 years (4-101 years). Same to the similar studies, the majority of these isolates were *B. fragilis* (58.3%), followed by *B. thetaiotaomicron* (19.8%), *B. vulgatus* (6.5%), *B. ovatus* (6.0%), *P. distasonis* (3.8%), *B. uniformis* (2.8%); and other *B. fragilis* group species (*B. caccae, B. nordii, B. salyersiae, B. stercoris, B. cellulosilyticus, B. intestinalis, P. goldsteinii*) were also identified in low rates (0.3-1%, total: 2.8%) (Figure 1). The most common sample types were wound (44.8%) and intraabdominal samples (42.7%); while extraabdominal abscess (3.8%), blood culture (4.2%) and other types (gynaecological samples, middle ear, cerebrospinal fluid, pericardial fluid) were less frequent (0.25-0.75%, total: 4.5%) (Figure 2). Half of the strains were collected in Surgery, 12.7% in an Intensive Care Unit (ICU), 12.5% in Internal Medicine, 5.8% in Pediatrics, 5.0% in Obstetrics and Gynaecology, and the remaining samples were collected in other departments (1.0-4.5%).

Figure 1.: Species distribution of *B. fragilis* group isolates



Other species: ***B. cellulosilyticus, B. intestinalis, B. caccae, B. salyersiae, B. strecoris, B. nordii, P. goldsteinii***

Figure 2.: Distribution of the *B. fragilis* group isolates according to the sample types



IV.2.2. Antibiotic susceptibility tests

The data of antimicrobial susceptibility, the MIC ranges, the MIC_{50} and MIC_{90} values are summarized in Table 6. 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 overall resistance rate of 7.0% was found to meropenem. A sum of 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 resistance rates of the different species, 91.67-100% of the species were resistant to ampicillin. *P. distasonis* strains had the highest resistance rates to amoxicillin/clavulanic acid (26.67%) and cefoxitin (20.0%). 16.66% of the *B. ovatus* strains were resistant to meropenem; whilst all of the *P. distasonis*, *B. uniformis* and other *Bacteroides* species were susceptible to this drug. The highest resistance rate for clindamycin of 55.7% was found among *B. thetaiotaomicron*, whilst 25.75% of the *B. fragilis* isolates were resistant. A large proportion of the strains (94.75%) were susceptible to tigecycline, with only four strains being resistant to

this antibiotic agent. Chloramphenicol also remained very active against *Bacteroides* species; with 99.5% of all the strains fully susceptible, and no resistant isolates found (Table 6). Antibiotic susceptibility data values of the centres are given in Table 7. A significant difference among highly ampicillin resistant (≥64 mg/l) strains was observed between centre pairs: Centre 1 and 4 ($p<0.001$); Centre 2 and 4 ($p<0.001$); Centre 2 and 4 ($p=0.002$). Comparing the cefoxitin resistant rates of the centres, the difference was significant between Centre 3: (3.0%) and Centre 4 (13.0%) ($p<0.001$). We detected a relatively high difference in meropenem susceptibility data. A total of 28 meropenem resistant strains were found, 25 of them were *B. fragilis* (89.28%, 25/28). Interestingly, in Centre 4 we identified 11 *B. fragilis* (39.28%, 11/28) and one *B. ovatus* (3.57%, 1/28) meropenem resistant strains. All of these *B. fragilis* strains were identified as a member of the Division II isolate by MALDI-TOF MS and harboured the *cfiA* gene proved by RT-PCR. In Centre 4 we found 11 high-level-meropenem-resistant strains (MIC≥16 mg/l) and one-one *cfiA*-positive strains with MIC-values of 4 mg/l and 8 mg/l. In other centres the rate of meropenem resistant strains was lower (4.0-7.0%) (Table 8) and all of them were high-level-meropenem-resistant (MIC≥16 mg/l). A significant difference in meropenem resistance was observed between Centre 1 and 4 ($p<0.001$) (Table 7). Clindamycin resistance displayed a relatively strong geographical difference, which was significant between Centre 1 (48.0%) and Centre 3 (27.0%) ($p=0.003$). The highest resistance rate to tetracycline was found among the strains isolated in Centre 1 (63.0%); while for Centre 4 this rate was 74.0%; but the difference was not significant ($p=0.121$) (Table 7). With a correlation analysis a strong correlation ($p<0.05$) was observed with the following antimicrobial drug pairs on the rate of resistant strains: ampicillin and amoxicillin/clavulanic acid; cefoxitin and amoxicillin/clavulanic acid; tetracycline and tigecycline. We analyzed the antibiotic susceptibility data based on the clinical source, but we did not find any significant correlation (i.e. $p<0.05$).

Table 6.: Antimicrobial activities of antibiotics against *B. fragilis* group isolates

Antimicrobial agents	MIC (mg/ml)			% of isolates		
	Range	MIC_{50}	MIC_{90}	S	I	R
***Bacteroides fragilis* group**						
Ampicillin	2->256	64	>256	0.75	1.25	98
Amox/clav.	0.064-32	0.5	8	87	8.5	4.5
Cefoxitin	0.5-256	8	32	77	16.25	6.75
Meropenem	0.064-32	0.5	4	88.75	4.25	7
Clindamycin	0.064->256	2	>256	63.25	0	36.75
Metronidazole	0.064-16	0.5	1	99.75	0	0.25
Moxifloxacin	0.064-64	1	8	75	6.5	18.5
Tetracycline	0.125-256	16	64	28.75	6	65.25
Tigecycline	0.064-64	0.5	4	94.75	3.75	1.5
Chloramphenicol	0.125-16	4	8	99.5	0.5	0
***B. fragilis* (n=233)**						
Ampicillin	2-512	64	>256	0	0	100
Amox/clav.	0.064-32	0.5	4	91.87	6.43	1.7
Cefoxitin	1-256	8	16	90.56	6	3.44
Meropenem	0.064-32	0.5	8	84.98	5.15	9.87
Clindamycin	0.064->256	1	>256	74.25	0	25.75
Metronidazole	0.125-4	0.5	1	100.	0	0
Moxifloxacin	0.125-64	0.5	8	76.40	8.15	15.45
Tetracycline	0.125-256	32	64	25.75	3	71.25
Tigecycline	0.064-32	0.5	4	94.85	4.3	0.85
Chloramphenicol	0.25-8	4	8	100	0	0
***B. thetaiotaomicron* (n=79)**						
Ampicillin	2-512	128	>256	1.27	0	98.73
Amox/clav.	0.064-32	0.2	8	83.54	13.92	2.53
Cefoxitin	0.5-256	16	32	53.16	37.97	8.87
Meropenem	0.125-32	1	2	92.41	6.33	1.26
Clindamycin	0.064->256	8	>256	44.3	0	55.7
Metronidazole	0.064-4	0.5	1	100	0	0
Moxifloxacin	0.064-32	1	16	75.96	5.06	18.98
Tetracycline	0.125-256	16	64	40.51	5.06	54.43
Tigecycline	0.064-64	0,5	4	94.94	3.8	1.26
Chloramphenicol	0.125-16	4	8	98.74	1.26	0
***B. vulgatus* (n=26)**						
Ampicillin	4-512	128	>256	0	0	100
Amox/clav.	0.125-16	1	16	69.23	23.08	7.7
Cefoxitin	0.5-128	4	64	80.76	7.69	11.55
Meropenem	0.125-32	1	4	84.62	11.54	3.84
Clindamycin	0.064->256	4	>256	50	0	50
Metronidazole	0.125-2	0.5	1	100	0	0
Moxifloxacin	0.25-64	2	32	50	0	50
Tetracycline	0.125-64	16	64	19.24	7.69	73.07
Tigecycline	0.064-8	0.2	2	96.16	3.84	0
Chloramphenicol	0.5-8	4	8	100	0	0
***B. ovatus* (n=24)**						
Ampicillin	2-512	256	>256	8.33	0	91.67
Amox/clav.	0.064-32	2	16	79.16	12.5	8.33
Cefoxitin	2-128	32	64	41.67	41.67	16.66
Meropenem	0.125-32	1	16	75	8.34	16.66
Clindamycin	0.064->256	8	>256	45.83	0	54.17
Metronidazole	0.125-8	0.5	2	95.83	0	4.17
Moxifloxacin	0.25-32	1	32	79.16	0	20.84
Tetracycline	0.125-32	8	32	29.16	20.84	50
Tigecycline	0.064-8	0.2	4	95.83	4.16	0
Chloramphenicol	2-8	8	8	100	0	0

Table 6.: (Continued)

Antimicrobial agents	MIC (mg/ml)			% of isolates		
	Range	MIC_{50}	MIC_{90}	S	I	R
***P. distasonis*(n=15)**						
Ampicillin	**8->256**	**>256**	**>25**	**0**	**0**	**100**
Amox/clav.	**0.125-32**	**4**	**16**	**53.33**	**20**	**26.67**
Cefoxitin	**2-128**	**16**	**128**	**60**	**20**	**20**
Meropenem	**0.25-4**	**0.5**	**4**	**86.67**	**13.33**	**0**
Clindamycin	**0.5->256**	**4**	**>25**	**66.67**	**0**	**33.33**
Metronidazole	**0.25-2**	**0.5**	**1**	**100**	**0**	**0**
Moxifloxacin	**0.25-2**	**0.5**	**1**	**100**	**0**	**0**
Tetracycline	**0.25-32**	**16**	**32**	**20**	**20**	**60**
Tigecycline	**0.125-4**	**0.5**	**2**	**100**	**0**	**0**
Chloramphenicol	**4-8**	**8**	**8**	**100**	**0**	**0**
***B. uniformis*(n=11)**						
Ampicillin	**32->256**	**128**	**>25**	**0**	**0**	**100**
Amox/clav.	**0.125-2**	**0.25**	**2**	**100**	**0**	**0**
Cefoxitin	**1-64**	**8**	**32**	**81.8**	**9.1**	**9.1**
Meropenem	**0.25-4**	**0.5**	**1**	**90.9**	**9.1**	**0**
Clindamycin	**0.064->256**	**>256**	**>25**	**45.45**	**0**	**54.55**
Metronidazole	**0.125-0.5**	**0.5**	**1**	**100**	**0**	**0**
Moxifloxacin	**0.5-32**	**0.25**	**1**	**72.72**	**9.1**	**18.18**
Tetracycline	**0.125-32**	**8**	**32**	**54.55**	**18.18**	**27.27**
Tigecycline	**0.064-2**	**0.5**	**2**	**100**	**0**	**0**
Chloramphenicol	**4-8**	**8**	**8**	**100**	**0**	**0**
Other *Bacteroides* species*						
Ampicillin	**2->256**	**64**	**64**	**0**	**0**	**100**
Amox/clav.	**0.125-4**	**0.5**	**4**	**100**	**0**	**0**
Cefoxitin	**1-64**	**16**	**32**	**41.67**	**41.67**	**16.67**
Meropenem	**0.125-2**	**0.5**	**1**	**100**	**0**	**0**
Clindamycin	**0.125->256**	**4**	**>25**	**41.67**	**0**	**58.33**
Metronidazole	**0.125-2**	**0.5**	**1**	**100**	**0**	**0**
Moxifloxacin	**0.064-64**	**2**	**16**	**66.67**	**8.33**	**25**
Tetracycline	**0.25-128**	**16**	**128**	**16.67**	**8.33**	**75**
Tigecycline	**0.125-32**	**0.25**	**16**	**75.0**	**0**	**25**
Chloramphenicol	**0.25-16**	**4**	**8**	**91.67**	**8.33**	**0**

**B. stercoris* (1), *B. cellulosilyticus* (1), *B. caccae* (4), *B. intestinalis* (1), *B. salyersiae* (2), *B. nordii* (2), *P. goldsteinii* (1)

Amox/clav.: Amoxicillin/clavulanic acid

S: susceptible, I: intermediate, R: resistant

Table 7.: Antibiotic susceptibility of *B. fragilis* group isolates obtained from the different Hungarian centres

Antimicrobial agents	MIC (mg/l) range	% of isolates		
		S	I	R
CENTRE 1 (n=100)				
Ampicillin	**4-512**	**0**	**5**	**95**
≥64 mg/l				**62**
Amox/clav.	**0.064-16**	**92**	**5**	**3**
Cefoxitin	**1-128**	**77**	**16**	**7**
Meropenem	**0.125-32**	**95**	**1**	**4**
Clindamycin	**0.125-**	**52**	**0**	**48**
Metronidazole	**0.125-4**	**100**	**0**	**0**
Moxifloxacin	**0.25-32**	**71**	**7**	**22**
Tetracycline	**0.125-128**	**31**	**6**	**63**
Tigecycline	**0.125-32**	**89**	**7**	**4**
Chloramphenicol	**0.25-8**	**100**	**0**	**0**
CENTRE 2 (n=100)				
Ampicillin	**2-512**	**1**	**0**	**99**
≥64 mg/l				**57**
Amox/clav.	**0.064-32**	**89**	**9**	**2**
Cefoxitin	**1-128**	**77**	**19**	**4**
Meropenem	**0.25-32**	**92**	**3**	**5**
Clindamycin	**0.064-**	**63**	**0**	**37**
Metronidazole	**0.064-4**	**100**	**0**	
Moxifloxacin	**0.125-32**	**72**	**9**	**19**
Tetracycline	**0.125-256**	**27**	**10**	**63**
Tigecycline	**0.064-16**	**97**	**1**	**2**
Chloramphenicol	**2-16**	**98**	**2**	**0**
CENTRE 3 (n=100)				
Ampicillin	**2->256**	**2**	**0**	**98**
≥64 mg/l				**73**
Amox/clav.	**0.125-32**	**89**	**8**	**3**
Cefoxitin	**0.5-128**	**80**	**17**	**3**
Meropenem	**0.125-32**	**87**	**6**	**7**
Clindamycin	**0.064-**	**73**	**0**	**27**
Metronidazole	**0.064-16**	**99**	**0**	**1**
Moxifloxacin	**0.125-64**	**78**	**5**	**17**
Tetracycline	**0.25-128**	**35**	**4**	**61**
Tigecycline	**0.064-8**	**99**	**1**	**0**
Chloramphenicol	**0.125-8**	**100**	**0**	**0**
CENTRE 4 (n=100)				
Ampicillin	**16->256**	**0**	**0**	**100**
≥64 mg/l				**91**
Amox/clav.	**0.5-16**	**77**	**14**	**6**
Cefoxitin	**1-256**	**74**	**13**	**13**
Meropenem	**0.064-32**	**81**	**7**	**12**
Clindamycin	**0.064-**	**65**	**0**	**35**
Metronidazole	**0.125-4**	**100**	**0**	**0**
Moxifloxacin	**0.25-64**	**79**	**5**	**16**
Tetracycline	**0.125-128**	**22**	**4**	**74**
Tigecycline	**0.064-8**	**95**	**5**	**0**
Chloramphenicol	**4-8**	**100**	**0**	**0**

CENTRE 1: Semmelweis University, Budapest; CENTRE 2: SYNLAB Ltd., Budapest; CENTRE 3: University of Debrecen; CENTRE 4: University of Szeged

Amox/clavulanic acid, S: suscpetible, I: intermediate, R: resistant

Table 8.: Meropenem MIC values of *cfiA*-positive and–negative *B. fragilis* and non-fragilis *Bacteroides* isolates

	Meropenem MIC (mg/l)	CENTRE 1	CENTRE 2	CENTRE 3	CENTRE 4
cfiA*-positive *B. fragilis	**≥16**	**1**	**2**	**3**	**11**
	8				**1**
	4				**1**
	<4	**1**			
cfiA*-negative *B. fragilis	**≥16**	**2**	**3**	**2**	
	8				
	4				
	<4				
Non-fragilis *Bacteroides*	**≥16**			**2***	**1****
	8				
	4				
	<4				

**B. ovatus, B. thetaiotaomicron* ***B. ovatus*

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

Among of investigated 400 *Bacteroides* strains 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 9). 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 summarized in Table 10. The *B. fragilis* SZ38 isolate harboured the *cfiA* gene with a high level resistance to ampicillin, amoxicillin/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 Ser82Phe 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.

Table 9.: Data of the MDR *Bacteroides* species strains

Isolate	Age & gender	Clinical presentation	Sample type	Department of isolation	MIC values (mg/l)										Treatment	OC
					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*	68 M	Diabetes mellitus, atherosclerosis, renal failure.	Decubitus	Internal medicine	>256	>16	32	1	>256	>8	32	32	4	8	WER, CLI, COL, VA	A
SZ4 *B. vulgatus*	64 M	Diabetic foot	Wound	Surgery	>256	>16	128	4	1	0.25	>32	16	0.25	4	CIP, MTZ	D
SZ9 *B. ovatus*	73 F	Appendicitis	Intraabdo-minal fluid	Surgery	>256	16	32	16	>256	0.5	32	32	0.5	8	CXM, MTZ, CIP	A
SZ34 *B. vulgatus*	63 M	*C. difficile* colitis	Autopsy	Pathology	>256	>16	128	4	>256	0.5	32	8	0.125	4	ND	D
SZ35 *B. thetiota.*	67 F	Abrasion	Intrauterine device	Obst. & Gynecol.	>256	16	128	2	>256	0.5	1	16	0.25	8	No antibiotic treatment	A
SZ38 *B. fragilis*	76 M	Diabetic foot, atherosclerosis, leg amputuation	Wound	Surgery	>256	>16	128	16	8	0.5	8	32	0.5	4	MER	D

AMP: Ampicillin, AMC: Amoxicillin/ clavulanic acid,, FOX: Cefoxitin, MER: Meropenem, CLI: Clindamycin, MTZ: Metronidazole, MOX: Moxifloxacin, TET: Tetracycline, TIG: Tigecycline, CHO: Chloramphenicol, VA: vancomycin, COL: Colistin, CIP: Ciprofloxacine, CXM: Cefuroxime, OC: Outcome, A: Alive, D: Deceased, M: male, F: female, aEUCAST: breakpoitns bCLSI breakpoits, ND: No data, Obst. & Gynecol: Obstertics and Gynaecology

Table 10.: RT-PCR results of the MDR *Bacteroides* species strains

Strain	*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: 1.2 kb regulator region, D/IS: deletion or other IS-element

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 melting curve analysis also differentiated between the *bft-1* and *bft-2* isotypes here, the average temperature of the *bft-1*-positive strains was 80.1±0.4°C and that of the *bft-2*-positive strains was 81.2±0.2 °C (Figure 3). The separation of *bft*-1 and *bft*-2 obtained from by the melting-cure analysis was confirmed by the sequencing of three *bft-1* and three *bft-2* positive *B. fragilis* isolates, which were randomly selected during the experiments. 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. During this study, a rare *B. fragilis* isolate was also found that originated from an abscess sample (*B. fragilis* SZ54) which contained the *cfiA* and the *bft-1* allele simultaneously. 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. Here, the distribution of the C10 protease genes was the following: 38 strains harboured *bfp1*, 58 isolates contained *bfp2* gene; while 17 isolates proved positive for *bfp3* and no *bfp4* positive strain was detected. Nine strains simultaneously harboured *bfp1*, *bfp2* and *bfp3* genes; 22 proved positive for *bfp1* and *bfp2;* while five isolates contained *bfp2* and *bfp3*; and one isolate proved positive for *bfp1* and *bfp3* (Table 11). 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 (Table 11). Among the *cfiA*-positive isolates, three harbouring *bfp1* and two *bfp3* were identified; while among the *cfiA*-negative strains 35 proved positive for *bfp1*, 56 for *bfp2* and 17 for *bfp3* (Table 12). Looking for significant positive or negative correlations among the genes investigated among the 72 selected *B. fragilis* isolates. None of the 63 *fpn*-positive isolates harboured the *cfiA* gene, so a significant negative correlation was demonstrated between *cfiA* and *fpn* ($p<0.000$) genes. A significant positive correlation was observed between the *bfp2* and *fpn* genes ($p=0.0000803$) and a negative correlation was found between the *bfp2* and *cfiA* genes ($p=0.011$). These new findings were quite expected and in the literature very little data can be found concerning the prevalence or distribution of these genes together.

Figure 3.: Melting curve analysis of *bft-1* and *bft-2* positive *B. fragilis* strains

Tm of *bft-1* positive strains↓ ↓Tm of *bft-2* positive strains

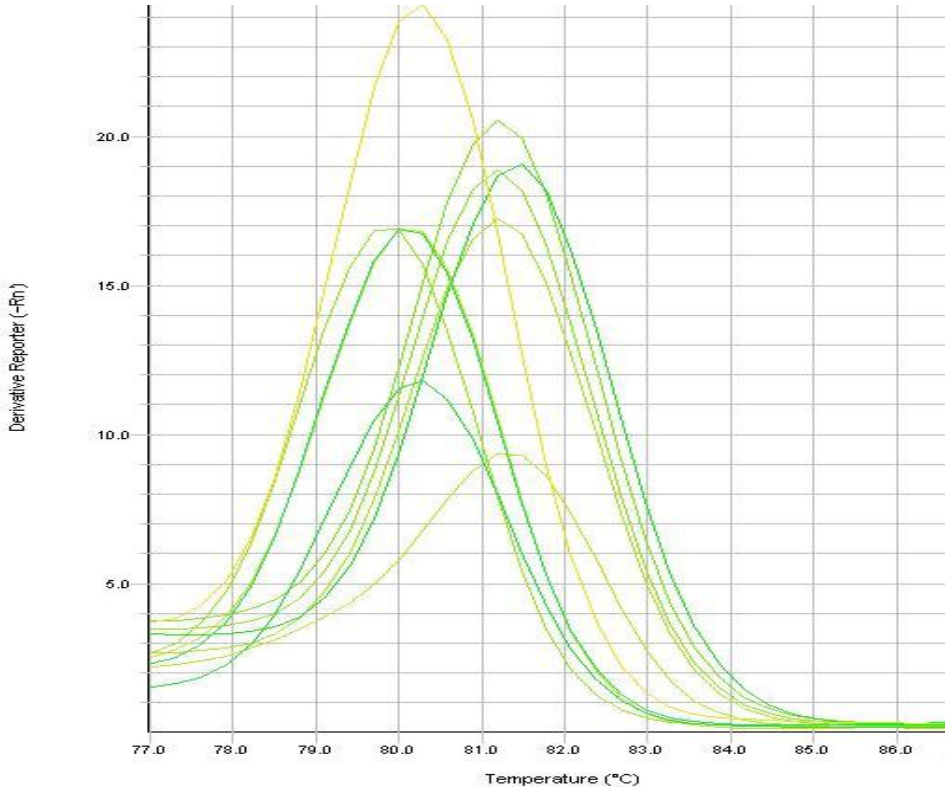


Table 11.: Distribution of *fpn* and *bfp1-4* genes among a subset of *bft*-positive and *bft*-negative *B. fragilis* isolates

Strains	**Number of *bft*-positive strains (n=26)**	**Number of *bft*-negative strains (n=46)**
***bfp1*-positive**	12	26
***bfp2*-posititve**	19	39
***bfp3*-positive**	7	10
***bfp4*-positive**	0	0
***bfp1- and bfp2*-positive**	3	19
***bfp1*- and *bfp3*-positive**	0	1
***bfp2*- and *bfp-3*-positive**	0	5
***bfp1*-, *bfp2*- and *bfp3*-positive**	6	3
fpn-positive	**24**	**36**

Table 12.: Distribution of *fpn* and *bfp1-4* genes in *cfiA*-positive and *cfiA*–negative *B. fragilis* strains

Strains	*cfiA*-positive	*cfiA*-negative
***bfp1*-positive**	3	35
***bfp2*-posititve**	2	56
***bfp3*-positive**	0	17
***bfp4*-positive**	0	0
***bfp1- and bfp2*-positive**	0	22
***bfp1*- and *bfp3*-positive**	0	1
***bfp2*- and *bfp-3*-positive**	0	5
***bfp1-, bfp2*- and *bfp3*-positive**	0	9
***fpn*-positive**	**0**	**63**

V. DISCUSSION

V.1. VALIDATION OF MALDI-TOF MS METHOD FOR THE IDENTIFICATION OF DIFFERENT *B. FRAGILIS* GROUP ISOLATES

As several studies demonstrated the increasing rate of antibiotic resistant strains among anaerobic bacteria adequate species-level identification is getting very important [78,96-98]. The traditional biochemical and the commercially available automatized methods have some limitations, for example the discrimination ability of biochemically similar strains is not sufficient. On the other hand, the results of identification may depend on the proper anaerobic environment and the deposited species in the library. Rapid ID 32A (bioMérieux, France), as well as some other tests, 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. According to the literature data, the correct identification with preformed enzyme diagnostic kits is only 78-79% of the *B. fragilis* group isolates [90]. Another disadvantage of the biochemical test can be the length of incubation time (rapid ID 32A: 4 h; API 20 A /bioMérieux, France/: 24 h; Remel rapid ID ANA II /Thermo Fisher Scientific, USA/: 4 h) and different incubation conditions, depending on the kind and the principle of kits. 16S rRNA gene sequencing is the most accurate method, but it's 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. According to the *dnaJ, gyrB, hsp60, recA, rpoB* and 16S rRNA gene sequencing data the phylogenetically related *Bacteroides* species classify to clades, e.g. species pairs: *B. intestinalis/B. cellulosilyticus, B. nordii/B. salyersiae, and B. ovatus/ B. xylanisolvens* [100]. 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. In the case of *Bacteroides* isolates SY23 (*B. vulgatus/B. fragilis*), SY53 (*B. ovatus/B. thetaiotaomicron*) and SE33 (*B. thetaiotaomicron/B. fragilis*) we accepted the 16S rDNA sequencing results. Culebras *et al.* reported that the accurate, species-level identification of *Bacteroides* strains with MALDI-TOF MS system is 87% in comparison with 16S rDNA sequencing method [90]. On the other hand the rate of correct identification with rapid ID 32A method was 52.3% [78]. Nagy *et al.* reported that the unequivocal identification rate of *Bacteroides* isolates was 98.6% of with MALDI-TOF

MS [8]. According to the data of study by Handal *et al.*, the species-level identification of *Bacteroides* and gram-positive anaerobic cocci blood culture isolates is 86.6% by MALDI-TOF MS [92]. We proved the superiority of MALDI-TOF MS system to automated biochemical tests. For validation of the method we applied three parallel measurements with the same conditions (strains, chemicals). Good reproducibility (94.75%) of MALDI-TOF MS identification method of *Bacteroides* species was proved.

V.2. EPIDEMIOLOGY OF ANTIBIOTIC SUSCEPTIBILITY

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 in fact Nagy *et al.* reported a similar result (97.4%) [78]. Only 4.5% of our isolates were resistant to amoxicillin/clavulanic acid, while Nagy *et al.* reported a rate of 8.7% [78] and Wybo *et al.* a rate of 14.0% [101]. A total of 6.75% of the strains exhibit a resistance to cefoxitin, which is much lower than reported in previous surveys (15.2-17.2%) [78,102]. The rate of cefoxitin resistance depends on the different species: for instance, 3.44% of the *B. fragilis* and 20.0% of the *P. distasonis* strains were resistant. This finding is in agreement with Snydman *et al.*, who found that 3.7% of the *B. fragilis* and 14.7% of the *P. distasonis* were resistant to cefoxitin [103]. In general, carbapenems have high activity against anaerobic bacteria, but the resistance to these drugs is increasing [96,97]. We confirmed this observation, as an overall species resistance level of 7.0% was observed to meropenem, with 9.87% of *B. fragilis* strains resistant to meropenem. A meropenem resistance rate for the *B. fragilis* group isolates of 0.5% was reported in an American study [98], and in Europe it was 1.3% [93]; however, Liu *et al.* found a resistance rate of 12% of *B. fragilis* strains in Taiwan [96]. Studies have reported a prevalence of *cfiA*-positivity of between 2.4 to 5.7% [104-106]; and of our 233 *B. fragilis* strains, 20 harboured the *cfiA* gene (8.58%). The difference of the meropenem resistance rates among the centres can be the different prevalence of the *cfiA* gene and the local antibiotic administration. According to the literature, in case of *cfiA*-negative *B. fragilis* strains the background of the meropenem resistance can be the alteration of the PBPs and their consequent poor affinity to β-lactams or decreased permeability [4]. We noticed an elevated overall resistance level of 36.75% to clindamycin, which varied among the different species. In fact it was lowest among *B. fragilis* (25.75%) and highest in the case of *B. thetaiotaomicron* (55.7%). Others have reported a clindamycin resistance rate of between 27 and 37.6% [78,107,108]. We found that clindamycin resistance displayed a relatively strong geographical difference, which is concordant with the results published by Nagy *et al.*: in the Southern European countries the

mean rate of clindamycin resistant strains was 37.6%; however, in Northern Europe it was found to be 81.4% [78]. Despite the frequent usage of metronidazole, this drug still shows excellent activity against *Bacteroides* isolates, only one strain was found to be resistant to it (0.25%). The overall resistance rate to metronidazole among *Bacteroides* isolates remains low (<1%) worldwide [57,78,102]. Among the different *Bacteroides* species the moxifloxacin resistance rate varies considerably; all of the *P. distasonis* (n=15) isolates were susceptible, but 15.45% of the *B. fragilis* (n=233) and 50.0% of the *B. vulgatus* (n=26) strains were resistant to moxifloxacin. Considerable differences in moxifloxacin susceptibility between species was observed by Snydman *et. al.*: the resistance rate varied from 38.9% in *P. distasonis* to over 70.0% of *B. ovatus*, *B. vulgatus* and other *Bacteroides spp.* (*B. caccae*, *B. eggerthii* etc.) [103]. Differences were observed in the susceptibility to clindamycin, cefoxitin and moxifloxacin among strains isolated in different regions. Nagy *et al.* reported significant regional differences of the rates of moxifloxacin resistance strains from Southern (92.45%) and Northern European (70.1%) countries [78] and we found particular geographical differences (Centre 3: 3% vs. Centre 4: 13%) in Hungary. We detected an overall resistance rate to tetracycline of 65.25% but there was also a great variation; with 27.27% of the *B. uniformis* (n=11) isolates, and 75.0% of the other *Bacteroides* isolates (n=12) resistant to this drug, and an overall rate of 65.25%. Tigecycline was very active, with only three resistant strains isolated (1.5%), which is consistent with the results published by Nagy *et al.* (1.7%) [78]. The effectiveness of chloramphenicol remained excellent, and with the exception of one intermediate susceptible strain all of the isolates were susceptible to chloramphenicol. Other studies confirm our general findings: Wybo *et al.* reported a susceptibility of 99.0% of 2004 [101], and Nitzan *et al.* a susceptibility of 98.5% among anaerobic isolates to chloramphenicol [109]. Only a limited comparison can be made between this data and historic Hungarian *Bacteroides spp.* susceptibility data. According to the data reported by Nagy *et al.*, the level of clindamycin resistance increased from 23.0% to 36.75% and moxifloxacin from 13.6% to 18.5%, but interestingly the level of resistance to amoxicillin/clavulanic acid decreased from 15.0% to 4.5% and cefoxitin from 24.0% to 6.75% (Table 13) [77,78]. The comparison of data of present and previous Hugarian studies is quite difficult because of the different methods (microbroth dilution [77] vs. agardilution [78]), different breakpoints and number of isolates. The background of decreased amoxicillin/clavulanic acid and cefoxitin resistance rate may be the different collection sites, change of the antibiotic usage, the different number of the isolates.

Table 13.: Comparison of previous and present Hungarian data of *B. fragilis* group isolates

Antimicrobial agents	Percentage (%) of resistance strains at different timepoints		
	1992 (n=200) [78]*	2010 (n=100) [79]**	Present study (n=400)
Ampicillin	**97**	**100[b]**	**98.00**
Amoxicillin/clavulanic acid	**ND**	**15[b]**	**4.50**
Cefoxitin	**11**	**24[a]**	**6.75**
Meropenem	**ND**	**ND**	**7.00**
Clindamycin	**23**	**27[b]**	**36.75**
Metronidazole	**0**	**1[b]**	**0.25**
Moxifloxacin	**ND**	**13.6[a]**	**18.50**
Tetracycline	**65**	**ND**	**65.25**
Tigecycline	**ND**	**1.7*** **	**1.50**
Chloramphenicol	**0**	**ND**	**0**

ND: No data

*Method: microbroth dilution, Resistance breakpoints: CLSI (formarely: NCCLS)

**Method: agar dilution, Resistance breakpoints: [a]CLSI (formarely: NCCLS) and [b]EUCAST

***Breakpoints published by Nagy *et al.* [79]

We observed a strong correlation ($p<0.05$) among the following three pairs: ampicillin and amoxicillin/clavulanic acid; cefoxitin and amoxicillin/clavulanic acid; and tetracycline and tigecycline. In the background there is a common antibiotic resistance mechanism: the *β*-lactamase production is the most common resistance mechanism for β-lactame antibiotics among *B. fragilis* isolates; more than 90% of the isolates express at least one *β*-lactamase gene [44-46]. Currently no valid, exact data about antibiotic prescribing practices are available in Hungary. The background of the reduction of cefoxitin resistance rate can be its very low usage. Interestingly, the consumption of amoxicillin/clavulanic acid is high and this was the first choice in *Bacteroides* infections, the resistant rate remained quite low. The rational restriction of antibiotics can help the control of other diseases, e.g. *C. difficile* infection. In the past decade, the number of reports of β-lactam/β-lactamase inhibitor combinations, cefoxitin, moxifloxacin, tetracycline and clindamycin resistant *B. fragilis* group isolates has increased worldwide [110,111]. The reasons for different resistance patterns maybe due to local antimicrobial

chemotherapy administration, the distribution of antibiotic resistance genes, the variation between susceptibility testing methods, the differences in the interpretative breakpoints or the complete lack of them.

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

To date, MDR *Bacteroides* isolates have been rarely published. In the past decade, some cases were published from the US [60,79], the UK [80], Greece [81], Japan [82] and Denmark [83]. The case of an American soldier was also published, who suffered serious injuries in Afghanistan [84]. 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, 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 [85]. In the upstream region of the *cfxA* gene, special genetic elements (IS*614B*, Tn4555 and Tn4351) can be usually detected, (which were described earlier by Garcia *et al.* and Sóki *et al.* [86,112], 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 and harboured *cfiA, nimA, nimD, nimE, nimJ, tetQ, ermB, ermF, linA2* (clindamycin resistance), *cepA, cfxA, bexB* [109]. *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 [108]. The novelty of this study is that we demonstrated a relatively significant incidence 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. The background of the significant incidence 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.

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

The asymptomatical carrying rate of ETBF strains in the gut has been shown to be 6.2-20.0% [113-116], while the prevalence of ETBF isolates among extraintestinal *B. fragilis* strains lies between 18.5-38.2% [113,117,118]. The percentage of enterotoxin producing *B. fragilis* strains isolated from blood cultures was also significant. Claros *et al.* investigated 63 *B. fragilis* isolates obtained from septic patients and the rate of the ETBF strains was found to be 19.0% [119]; however, Kato *et al.* reported a 28.1% rate of fragilysin production among blood culture isolates [120]. Our data showed that the majority of the 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* (62.5%, 10/16), while 25.0% (4/16) harboured the *bft-2* isotype and 12.5% (2/16) harboured the *bft-3* isotype [23]. Our data revealed a slightly similar distribution of the different isotypes, but the apparent lack of *bft-3* might be due to the different geographical distribution of the alleles. The metalloprotease activity of these isolates on the cell culture of HT29/C1 cells suggests that there is a potential invasivity of the *bft*-positive *B. fragilis* isolates in different types of infections [121]. In order to identify the three *bft* isotypes and separate them, PCR-RFLP was applied. However, using the RT-PCR to detect the genes, we found that melting curve analysis was also able to distinguish between the *bft-1* and *bft-2* isotypes. To confirm the validity of the melting analysis, the sequencing of randomly chosen three *bft-1* and three *bft-2* harbouring *B. fragilis* isolates was performed. The complete identity using the published sequences in the Genbank of the *bft* subtypes helped confirm the reliability of this technique. A good correlation was also observed between the results obtained from the melting curve analysis and those got via the PCR-RFLP, which were then used to differentiate between the *bft*-1 and *bft*-2 isotypes. We investigated the correlation between *bft* and *cfiA* genes as well. The high discrimination power of MALDI-TOF MS between *B. fragilis* Division I and II was demonstrated by Nagy *et al.* and Wybo *et al.* [9,121]. To verify earlier results, all the 200 *B. fragilis* isolates were investigated with the MALDI-TOF Biotyper 3.1 software *cfiA* identification project file developed by Bruker Daltonik and used by Fenyvesi *et al.* [91]. Nineteen isolates (9.5%) were placed into Division II and RT-PCR confirmed the presence of the *cfiA* gene in all of the *B. fragilis* strains belonging to Division II. Our results indicated a slightly higher rate of *cfiA*-harbouring *B. fragilis* strains compared to the baseline range of 2.0-8.85% [47,122,123]. Among the 200 isolates we were

able to identify a strain called *B. fragilis* SZ54 that harboured the *cfiA* and *bft* genes simultaneously, which is a rare finding, and only one similar strain was described earlier by Soki *et al.* [123]. In the cysteine protease families, seventy two cystein protease families have been identified so far of these, 43 families belong to nine exclusive cysteine protease superfamilies (clans) namely, CA, CD, CE, CF, CH, CL, CM, CN and CO [124]. These enzymes can be found in plants, animals, fungi, humans, bacteria and parasites as well. Four members of the C10 family cysteine proteases belonging to the CA superfamily were discovered in *B. fragilis* strains. It has been suggested that these enzymes might be involved in the pathogenesis of inflammatory bowel disease or irritable bowel syndrome [28]. A total of 34 strains harboured two isotypes, while nine isolates contained simultaneously three *bfp* isotypes. Among the *bft*-positive and –negative *B. fragilis* strains investigated, the *bfp2* isotype was the most prevalent and a positive correlation was found between the *bfp2* gene and *fpn* gene. Recently has been demonstrated that *B. fragilis* strains produce a special protease called fragipain (Fpn), which is a member of the C11 family (belonging to the CD clan). Fragipain is required for the activation of fragilysin and Choi *et al.* hypothesised the possible role of fragilysin in the progression of sepsis [28]. 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. Morever, fragipain might have a further role in the cell function and pathogenesis in the sepsis, because members of the CD clan have several functions, these being cell proliferation, the regulation of cell death pathways, inflammation, the clearance of insoluble aggregates, virulence etc. [125]. In summary, in this study we investigated the *bft* gene in *B. fragilis* that was isolated from various extraintestinal clinical samples and we looked for correlations among *bft, bfp1-4, fpn* and *cfiA* genes. We found quite similar rate of the *bft* harbouring strains (13.0%) compared with our previous study from 2006 (8.7%) [29]. The first study from our Institute reported higher *bft* carriage rate (25.3%), but the strains were investigated by using only the cytophatic effect on HT29/C1 cell line, which is a very subjective method [113]. The novelty of our present study was the particular molecular investigation of the *bft, bfp1-4, fpn* and *cfiA* genes, where we found positive and negative statistical correlations.

VI. CONCLUSIONS

The aims of our study were: to validate of MALDI-TOF MS method for identification of *B. fragilis* group isolates, to evaluate the local epidemiology of clinically relevant *B. fragilis* group isolates from clinical microbiological centres, to investigate the molecular backgound of MDR *Bacteroides* strains, to measure the the incidence of *B. fragilis* enterotoxin gene and its isotypes, C10, C11 cyteine protease and *cfiA* genes among *B. fragilis* strains.

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 rates of cefoxitin, tetracycline and moxifloxacin resistance depend 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 incidence 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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