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

Ph.D. Thesis

Ph.D. candidate: Zsuzsa Eitel, M.Sc.
Ph.D. supervisor: Elisabeth Nagy M.D., Ph.D., D.Sc.

Institute of Clinical Microbiology
Doctoral School of Interdisciplinary Sciences

Szeged, 2016

LIST OF PUBLICATIONS

This dissertation is based on the following publications:

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

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

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

IV. Székely E, **Eitel** Z, Molnár S, Szász IÉ, Bilca D, Sóki J: Analysis of Romanian *Bacteroides* isolates for antibiotic resistance levels and the corresponding antibiotic resistance genes. Anaerobe, 31:11-14, 2015.(IF: 2.48)

V. Nagy E, Justesen US, **Eitel** Z, Urbán E: Development of EUCAST disk diffusion method for susceptibility testing of the *Bacteroides fragilis* group isolates. Anaerobe, 31:65-71, 2015. (IF: 2.48)

CONTENTS

ABBREVIATIONS

BFT	*Bacteroides fragilis* toxin
CLSI	Clinical and Laboratory Standards Institute
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetraacetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IS	insertion sequence
kb	„kilobase pair”
LPS	lipopolysaccharide
MATE	multidrug efflux transporter pumps
PCR	polymerase chain reaction
PBP	penicillin-binding protein
PFGE	pulsed-field electrophoresis
rRNA	ribosomal ribonucleic acid
RT-PCR	real-time polymerase chain reaction

1. INTRODUCTION

1.1. Description of *Bacteroides fragilis* group strains

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

In addition, *Bacteroides* are opportunistic pathogen organisms. Although they represent only 0.5% of the bacterial population occur in the faeces, *B. fragilis* group strains are the most frequently isolated species in anaerobic infections. Practically pathogen *Bacteroides* strains can be isolated from infections of any part of the human body [Finegold, 1995]. They cause severe intra-abdominal infections, postoperative wound, skin and soft-tissue infections together with other anaerobic and aerobic bacteria, or they might also be the causative agents of bacteremia. The most virulent *Bacteroides* species is the *B. fragilis*. This organism is responsible for the 80% of the infections caused by the members of the *Bacteroides* genus and are considered the most virulent strain in *B. fragilis* group strains. The *B. fragilis* is found in many anaerobic infections with an associated mortality of more than 19% in bacteremia and if a documented *B. fragilis* infection is left untreated, the mortality rate is reported to be about 60% [Goldstein, 1996]. Healthy individuals rarely become infected; the infection is usually due to a pre-existing underlying disease or after any intraabdominal surgery procedures. *B. fragilis* and related species possess numerous virulence factors, antibiotic resistance genes, which enables a high degree of ability to infect the human body. The *Bacteroides* isolates require glucose, haemin, minerals and vitamin B_{12} and are resistant to 20% bile.

In 1898, Veillon and Zuber was the first who described *B. fragilis* as *Bacillus fragilis* [Veillon and Zuber, 1898]. In 1919, the bacterium was transferred to the *Bacteroides* genus by

Castellani and Chalmers [Castellani and Chalmers, 1919]. Nowadays, the genus comprises of Gram-negative, obligate anaerobic, rod-shaped bacteria. The inclusion or exclusion of species within the genus *Bacteroides* changes continuously, thanks to the new, more reliable molecular biological procedures (DNA-DNA hybridization, 5S-16S ribosomal RNA (ss-rRNA) gene sequencing and whole genome sequencing) [Dix *et al.*, 1990; Socransky *et al.*, 1994]. Currently we know more then 20 *Bacteroides* species and 5 *Parabecteroides* species, for example *Parabacteroides distasonis* (formerly *Bacteroides distasonis*), and *Parabacteroides merdae* (formerly *Bacteroides merdae*). [Wexler, 2007].

1.2. Virulence factors of *B. fragilis*

1.2.1. The bacterial capsule

The *B. fragilis* strains may have three types of morphologically different surface structure: 1) a large capsule, which can be detected by light microscope or electron microscope, 2) a small capsule, which also can be examined by light microscope or electron microscope and 3) an electron-dense layer which can only be studied by electron microscopy (non-capsulated variant) [Patrick, 1997]. Experiments in mice and rats infected with *B. fragilis* strains carrying capsule, is followed by intra-abdominal ulcers observed in experimental animals [Onderdonk *et al.,* 1977]. *B. fragilis* is the anaerobic bacterium, which was shown to induce abscess formation, as the only organism responsible for the infection and the injection of capsules alone was sufficient to induce abscess formation [Kasper *et al.*, 1979]. Animal experiments have also shown that systemic injection the capsule material alone can develop protection against the formation of abscesses [Kasper *et al.,* 1979]. This is a T-cell dependent immunity. Examination of the *B. fragilis* NCTC 9343 strain revealed that the capsule is made up of two distinct polysaccharide complexes which have been assigned PS A and PS B. PS A is made up of tetrasaccharide units that carry a positively charged amino group and a negatively charged pyruvate substituent and a PS B is made up of repeating units hexasaccharid having two negative and a positive charge. The capsule can be observed in other *Bacteroides* species as well, it plays a role not only in the formation of abscesses, but protects bacterial cells from phagocytosis by granulocytes [Pantosti *et al.,* 1993].

1.2.2. The role of endotoxin / lipopolysaccharide (LPS)

For a long time it was thought that the *Bacteroides* LPS is biologically inactive, however,

in vitro experiments have shown that they do have biological activity [Delahooke *et al.,* 1995; Szöke *et al.,* 1997]. Endotoxin release due to the effect of antibiotic therapy is several times stronger in the case of *B. fragilis* strains than observed with other *Bacteroides* species [Rotimi *et al*., 2000]. In consequence of the TNF-inducing activity of *B. fragilis* and related species and the about 100 times larger frequency of *Bacteroides* relative to *Enterobacteriaceae* in the colon, *Bacteroides* LPS may play an important and previously overlooked role also in endotoxic shock. The TNF and Il-6 stimulatory activities of intact *Bacteroides* cells and their isolated LPS have also been demonstrated [Szöke *et al.,* 1997]. This may explain the importance of *B. fragilis* and related species in monobacterial or mixed infections.

1.2.3. Enterotoxin production

The first suggestion was that some *B. fragilis* strains are toxigenic (so-called enterotoxigenic *B. fragilis,* ETBF) during studies of the epidemic diarrheal disease of lambs [Myers *et al.,* 1984; 1985]. Since then further studies have confirmed that certain *B. fragilis* strains secrete a toxin (termed *B. fragilis* toxin/ BFT or fragilysin) [Myers *et al.*, 1987; Szöke *et al.,* 1997] which is a metallo-protease [Moncrief *et al.*, 1995], capable of cleaves E-cadherin protein in the zonula adherens of the intestinal cells, resulting in rearrangements of the actin cytoskeleton of the epithelial cells and loss of tight junctions [Wu *et al*., 1998] These changes are leading to diarrhea in calves, foals, piglets, rabbits and at the end of the 1980s it was also proven in humans [Myers *et al.,* 1987]. BFT is secreted by enterotoxigenic *B. fragilis* strains, which encode three isotypes of BFT on a distinct *bft* locus, carried on a 6-kb genome segment unique to these strains, called the *B. fragilis* pathogenicity island. There is evidence that the pathogenicity island with the enterotoxin gene is contained within a novel conjugative transposon [Franco, 2004]. This pathogenicity island is flanked by genes encoding mobilization proteins and may thus be transmissible to non-toxigenic strains [Franco *et al.*, 1999; Wexler, 2007].

1.2.4. Aerotolerance

The *Bacteroides* species has been shown to be aerotolerant, capable of surviving more than 48 hours of exposure to O_2, and this may play important role in the initiation or persistence of infection [Tally *et al.*, 1975]. In *B. fragilis*, catalase (*katB*), superoxide dismutase (*sod*), alkyl hydroperoxide reductase (*ahpCF*), and Dps (nonspecific DNA binding protein; dps) production has been proven to increase extremely during exposure to oxidative stress [Gregory *et al*., 1977;

Gregory, 1985]. The ability to survive in a relatively long time in environments, which contain oxygen, may help the *Bacteroides* strains to withstand oxidative stress during the process of transmission between hosts and for the initial steps in colonization of the intestinal tract of newborns [Smalley *et al.*, 2002] and also to survive in the clinical specimens during the transport to the laboratories [Finegold *et al*., 1992]

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

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

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

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

In case of some β-lactam antibiotics such as penicillin, ampicillin, but also in case of erythromycin, tetracycline and clindamycin a constantly increasing resistance was observed, because of this, the therapeutic usage of these drugs can be recommend only after the antibiotic susceptibility testing of the isolates has been done [Rasmussen *et al*., 1993; Nagy *et al*., 2011]. The carbapenems (imipenem, meropenem), β-lactam/β-lactamase inhibitor combinations

(amoxicillin/clavulanic acid, piperacillin/tazobactam), the newer fluoroquinolones (moxifloxacin, trovafloxacin, gemifloxacin) and metronidazole are antibiotics which can most successfully used in empiric therapy of infections involving *B. fragilis* group isolates [Wadsworth-KTL 6th edition, 2002; Löfmark *et al*., 2010; Nagy *et al*., 2011]. The resistance level of *Bacteroides* strains to these antibiotics may vary depending on the geographical location where the strain was isolated [Nagy *et al.,* 2011]. The development of the current rather high resistance values to some antibiotics can be due to extensive and not always appropriate antibiotic usage similar to aerobic and facultative anaerobic bacteria [Rasmussen *et al.,* 1993; Edwards, 1997; Wexler, 2007]. In addition, nowadays increasing number of reports on multi-resistant clinical isolates of *Bacteroides* have been published [Rotimi *et al*., 1999; Wareham *et al*., 2005; Hartmeyer *et al*., 2012].

1.3.1. Resistance mechanisms to β-lactam antibiotics

The β-lactam antibiotics (e.g. penicillin G, ampicillin), are among the firstly discover antibacterial agents against which nowadays a major part of the bacteria possess resistance mechanisms. Among *Bacteroides* the most important mechanism of resistance against the β-lactam antibiotics is the enzymatic decomposition of the active agent. Previous research have shown that the 97% of *B. fragilis* strains in the United States [Aldridge *et al*., 1988] and 76% of *B. fragilis* strains in the United Kingdom [Edwards and Greenwood, 1992] produce β-lactamase enzymes (which may inactivate penicillin and in some extent ampicillin). Insertion sequence (IS) elements appear to cause in certain *Bacteroides* strains increased cephalosporin resistance as well by increasing the β-lactamase production of these strains.

The carbapenems are the broadest spectrum β-lactam antibiotics. Imipenem was the first member of this group, meropenem and the recently introduced ertapenem and doripenem are all active for *Bacteroides* strains. Therapeutic usage of them is especially important in mixed infections when other antibiotics are unable to eliminate the pathogens. For this reason, particular importance is the emergence of resistance among *B. fragilis* group strains against carbapenems [Edwards *et al*. 1999; Yamazoe *et al*., 1999; Sóki *et al*., 2000;].

The β-lactamases found in *Bacteroides* strains can be divided into 4 groups according to the scheme developed by Bush *et al*. (1995). Most common β-lactamases can be put into the "2e" group. They hydrolyze cephalosporins (better than the penicillins), do not act against cefoxitin, latamoxef or imipenem, and they are inhibited by cloxacillin, pCMB, clavulanic acid, sulbactam and tazobactam [Rasmussen *et al*., 1993]. This group includes the cephalosporinase enzyme encoded by *cepA* gene, which can be found also in ampicillin-resistant *Bacteroides* strains. The

second group of β-lactamase enzymes of *Bacteroides* belongs to "2d" group. These enzymes cause resistance against penicillin (including cloxacillin) and is inhibited by clavulanic acid and sulbactam [Rasmussen *et al*., 1993]. The enzymes that hydrolyze cefoxtin and moxalactam create the third group of β-lactamases. They are inhibited by clavulanic acid. The cefoxitin resistance gene, the *cfxA*, is located on a mobilized transposon (MTn*4555*) and able to spread between *Bacteroides* strains by conjugation [Smith and Parker, 1993]. Metallo-β-lactamases belong to the fourth group of the β-lactamases of *Bacteroides*. These enzymes are able to hydrolyze the cephamycins and carbapenems, are not inhibited by clavulanic acid, however they are efficiently inhibited by EDTA. Further studies (cloning, sequence and PCR analysis) of carbapenem-resistant *Bacteroides* strains showed that its gene is *cfiA* (or *ccrA*) and is located on the chromosomes [Edwards, 1997]. Previous studies showed that *cepA* and *cfiA* carrying *B. fragilis* strains proved to belong in two separate groups according to IS element content, genomic PCR typing, multilocus enzyme electrophoresis (MLEE) and pulsed-field gel-electrophoresis (PFGE), however these strains are not indistinguishable by usual biochemical tests [Podglajen *et al*., 1995]. Similarly, comparison of small-subunit ribosomal DNA sequences of some *B. fragilis* strains has also shown that they are separated into two subgroups, one in which the *cfiA* gene is carried (Division II), and one that consisted of *cfiA*-negative strains (Division I) [Podglajen *et al*., 1995; Ruimy *et al*., 1996]. The *B. fragilis* strains which belong to Division I carry the *cepA* and *bft* genes, while the Divison II group strains carry the *cfiA* gene [Gutacker *et al*., 2000].

The different permeability of the outer membrane (OM) also plays a role in resistance of *Bacteroides* against β-lactams. Although the low permeability of the membrane itself does not induce resistance, this requires additional production of β-lactamase or alteration in penicillin binding proteins.

The change in PBPs itself is also an effective mechanism of resistance against β-lactams, in the case of *Bacteroides* strains. The affinity of PBPs are reduced against some β-lactams such as piperacillin, cefoperazone, cefotaxime, cefoxitin and imipenem. [Edwards, 1997; Sóki *et al*., 2011].

1.3.2. Resistance mechanisms to clindamycin

The clindamycin resistance among *Bacteroides* is similar to the macrolide-lincomycin-streptogramin (MLS_B) resistance where a common mechanism mediate resistance to chemically different antibiotics. This resistance mechanism has been well studied among clindamycin-

resistant staphylococci and it is acting by methylation of one or two specific adenine nucleotides on 23S rRNA [Rasmussen *et al*., 1993]. The most common transferable plasmids that cause transfer of clindamycin resistance are the following: pBF4 (41 kb), pBFT (pCP1) (15 kb) and pB1136 (80 kb), in *Bacteroides* strains. The resistance genes occur on these transposons: the *ermF* gene on the Tn*4351* (pBF4) and the Tn*4400* (pBFTM10); and the *ermFS* on the Tn*4551* (pB1136) [Smith, 1987; Smith *et al*., 1998]. Furthermore, clindamycin resistance genes occur on chromosomal conjugative transposons. However, some other resistance mechanisms can be expected as well, since no cross-hybridization with the *ermF* gene sequence could be found among other clindamycin-resistant *Bacteroides* strains. [Rasmussen *et al*., 1993].

1.3.3. Resistance mechanisms to metronidazole

The first 5-nitroimidazole used clinically against anaerobic infection was metronidazole in the 1960s, and no metronidazole-resistant *Bacteroides* isolate was found until 1978 [Ingham *et al*., 1978]. One important and well-characterized type of resistance to metronidazole is associated with the presence of *nim* genes, which were described in 1994, and today we known 8 types of it (*nimA-I*) [Haggoud *et al*. 1994; Trinh and Reysset, 1996; Stubbs *et al*., 2000; Schapiro *et al*., 2004]. They are able to induce moderate to high-level metronidazole resistance, and may occur on four types of plasmids: pIP*417* (7.7 kb, *nimA*) pIP*419* (10 kb, *nimC*) pIP*421* (7.3 kb, *nimD*) and pBF*388c* (8.2 kb, *nimE*) and can also be found on the chromosome [Haggoud *et al*., 1994]. The *nim* genes are activated in all cases by IS elements and they encode a nitroimidazole reductase protein [Reysset, 1996; Carlier *et al*., 1997]. This type of resistance mechanism can be transferred by conjugation [Reysset *et al*., 1993]. The other type of resistance against metronidazole that results high metronidazole MICs, involves multiple chromosomal mutations and is not transferable. This resistance may be a sum of reduced nitroreductase activity, decreased uptake of the drug, increased lactate dehydrogenase and decreased pyruvate-ferredoxin oxydoreductase activities [Rasmussen *et al*., 1997]. Other mechanisms that may contribute to resistance to metronidazole in *Bacteroides* species include over-expression of multidrug efflux pumps [Pumbwe *et al*., 2007]. In addition, recent studies have shown that *B. fragilis* RecA protein overexpression may also causes resistance to metronidazole. This DNA repair protein has role in maintaining endogenous DNA stability and its contribution to resistance to metronidazole and other DNA damaging agents [Steffens *et al*., 2010]. Resistance to metronidazole is one of the rarest type of resistance among *Bacteroides* strains.

1.3.4. Resistance mechanisms to tetracyclines

The mechanism of action of these antibiotics is based on inhibition of the bacterial protein synthesis. The members of this group of antibiotics are among others tetracyclin, doxycycline and tigecycline, which latter is the first member of a new subgroup, namely, glycylcyclines. Tetracycline was a very effective antibiotic for treatment *Bacteroides* infections up to the late 60's, recently a great part of the *Bacteroides* isolates are resistant to this antibiotic. The increase of this resistance rate is due to the frequent presence of chromosomal conjugative transposons or Tc^R elements, which are carrying the tetracycline resistance gene (*tetQ*) [Salyers *et al*., 1995]. The mechanism of action of these proteins expressed by these genes (in addition to *tetM* and *tet36*) is based on ribosomal protection [Roberts, 1996]. The *tet36* gene was not present in human clinical and intestinal *Bacteroides* isolates, but was found in diverse bacterial genera including *Bacteroides* taken from swine manure [Whittle *et al*., 2003]. In addition, other resistance mechanisms against tetracycline were found in *Bacteroides* strains. One of them is the *tetX* gene, which could be found on Tn*4351,* on Tn*44000* and on some conjugative Tc^R elements [Rasmussen *et al*., 1993]. The product of *tetX* and *tetX1* genes is an FAD-dependent monooxygenase, which destroys tetracycline, but may also raise the MIC values of tigecycline [Shoemaker *et al*., 2001].

1.3.5. Resistance mechanisms to quinolones

BexA is an efflux pump, which is encoded by the *bexA* gene, a member of the multidrug and toxic compound extrusion class family (MATE) and has been described in *Bacteroides thetaiotaomicron* [Miyamae *et al*., 2001]. This efflux pump is one of the possible resistance genes responsible for developing resistance to the newer fluoroquinolones, including moxifloxacin of *B. fragilis* group strains. One further mechanisms of quinolone resistance have been identified in gram-negative organisms: mutations in DNA gyrase. A single point mutation in *gyrA* or in with the homologous *parA* has been shown to reduce susceptibility to fluoroquinolone [Deguchi *et al*., 1996; 1997].

1.4. The antibiotic susceptibility testing methods for anaerobes

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

1.4.1. Agar dilution method

Agar dilution method is the gold standard reference method for determination of MICs of antibiotics, that can be utilized to test the susceptibility of any anaerobic bacteria. This method has a wide variety of advantages: this is the most accurate way to measure the resistance of bacteria to antibiotics and up to 30 isolates (plus two controls) can be tested at once on a series of agar plates containing the double dilutions of an antibiotic. Accordingly, agar dilution is very useful method for testing several isolates simultaneously. Disadvantages are that is takes a lot of time, because of the agar plate preparation with the specific amount of antibiotic is time consuming, and the plates should be used as soon as possible because of the instability of some antibiotics. At the same time just one antibiotic can be tested on a series of plates and the investigation only one or few clinical isolates is a very wasteful and expensive procedure. Evaluation of the results may require experienced personal, but accurate MIC determination is possible. For these reasons this method is usually used for large evaluations of some groups of anaerobic bacteria in one time, whereas it is possibly the most accurate and reliable method [Philips *et al.*, 1992; Olsson-Liljequist and Nord, 1994; Betriu *et al*., 2008; Veloo *et al*., 2011]. Agar dilution method is recommended by CLSI as reference method for antibiotic resistance surveillances [CLSI, M11-A7, 2007].

1.4.2. Broth micro-dilution method

The broth micro-dilution method for determination of MICs is easier to perform than agar dilution, but has only been validated for antimicrobial susceptibility testing of *B. fragilis* group bacteria [CLSI, M11-A7, 2007]. The broth micro-dilution method can evaluate the susceptibility of multiple antibiotics simultaneously using the same microtiter plate. Nonetheless this method is not recommended to test the antimicrobial susceptibility of different slow growing anaerobic clinical isolates in the routine laboratories [CLSI, M11-A7, 2007; Jenkins and Schuetz, 2012].

1.4.3. Gradient diffusion method (E-test)

The E-test is a widely used method in microbiology diagnostic laboratories to determine the antibiotic susceptibility of the isolates based on MIC determination [Citron *et al.*, 1991]. This method is easy to perform and evaluate, but testing large numbers of isolates or a wide range of antibiotics could not be accomplished efficiently with this method. It requires less agar plates compared to the agar dilution method, but more than to use the semi-quantitative determination of antibiotic susceptibility of anaerobic bacteria by the disc diffusion method. This method is quite expensive, thus in the routine laboratories (at least in particular countries) it is used successfully to test the antibiotic susceptibility of clinical anaerobic isolates. Sometimes its applicability depends on the laboratory financial situation, unfortunately.

1.4.4. Disc diffusion method

The disc diffusion method for susceptibility testing of facultative and aerobic bacteria is accepted world-wide. It is a semi-quatitative method, but with careful standardization (of the antibiotic contents of the discs, the inoculum, the media used for culturing and the incubation time) the results (the inhibition zone diameters) correlate well with the MICs of the antibiotics tested. Most of the discussed available techniques for antimicrobial susceptibility testing of anaerobic bacteria based on MIC determination, although allowing testing of the majority of clinical isolates, are long, cumbersome, and costly for routine use in clinical laboratories. To use the disk diffusion method would be less expensive and easier to perform than any other antimicrobial susceptibility testing method based on MIC determination. Disk diffusion susceptibility testing of anaerobes has been evaluated in the past [Wilkins *et al*., 1972], but has not gained general acceptance [Sutter *et al*., 1972; 1973] especially not for the slow growing anaerobes. Recently the European Committee on Antimicrobial Susceptibility Testing (EUCAST) started to harmonize the disc diffusion method for antimicrobial susceptibility testing and

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

2. AIMS OF THE STUDY

The aims of this study were:

I. To evaluate the EUCAST disk diffusion method for susceptibility testing of a large number of *Bacteroides* strains by comparing disk diffusion susceptibility testing results with MICs determined by agar dilution or gradient test (E-test) for a wide variety of antibiotics suggested for treatment of anaerobic infections.

II. To determine the incidence of clinically important *cfiA* and *nim* genes among 640 *B. fragilis* group strains obtained from different European countries. Beside to test the two most important resistance genes we were also interested in the presence and distribution of the *bft* gene responsible for the toxin production in *Bacteroides* strains and the possible co-existence of the *bft* and *cfiA* genes among clinical isolates.

III. The incidence of a wide variety of other clinically significant antibiotic resistance genes were also tested among a subset of 161 of the previously tested 640 *B. fragilis* group isolates

IV. To study the co-occurrence of the detected resistance genes, among *B. fragilis* and non-fragilis *Bacteroides* isolates in connection with their resistance to antibiotics.

V. To study the antibiotic resistance and the resistance gene content of a recent collection of *B. fragilis* group isolates obtained from Romania (not included in the previous European surveillance)

3. MATERIALS AND METHODS

3.1. Bacterial strains and cultivation

Out of a big collection of different species belonging to *Bacteroides* and *Parabacteroides* genera 640 isolates were used during the different studies described in this thesis. The distribution of the species can be seen in **Table 1**. The strains were collected from 13 European countries for an antibiotic resistance surveillance in 2008-2009 [Nagy *et al*., 2011] and maintained in -80^{o}C in the Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary till usage for different studies described here. In addition, there were 53 *B. fragilis* group clinical isolates (36 *B. fragilis*, 7 *B. thetaiotaomicron*, 7 *B. ovatus* and 3 *B. vulgatus*) which were collected in the period of 2010 and 2013 at the Diagnostic Laboratory of the Emergency Department of the County Hospital at Targu-Mures, Romania as this country did not participate in the Europe-wide surveillance. Species identification was carried out previously by classical routine methods and confirmed by MALDI-TOF MS if identification was not acceptable [Nagy *et al.* 2009; Nagy *et al.* 2011]. Not all the isolates were used in all studies included in this dissertation. **Table 2.** shows the number of *B. fragilis* and non-fragilis *Bacteroides* isolates involved in the different studies.

Table 1. Distribution of the *Bacteroides* species investigated in these studies

Species	Number of strains
Bacteroides fragilis	486
Non-fragilis *Bacteroides*	154
Bacteroides thetaiotaomicron	54
Bacteroides ovatus	36
Bacteroides vulgatus	33
Bacteroides uniformis	8
Parabacteroides distasonis	7
Parabacteroides merdae	4
Bacteroides eggerthii	3
Bacteroides massiliensis	3
Bacteroides nordii	3
Bacteroides caccae	2
Bacteroides stercoris	1
Altogether	640

Table 2. Number of the *Bacteroides* strains used in different studies

Different studies	Number of the strains included		
	B. fragilis	Non-fragilis *Bacteroides*	Altogether
Looking for the *cfiA*, *nim* and *bft* genes (Paper I)	486	154	640
Testing of co-existance of *cfiA* and *bft* genes (Paper II)	486	0	486
Looking for a great variaty of other resistance genes (Paper III)	128	33	161
Testing of isolates from Romania (Paper IV)	36	17	53
Evaluation of disc diffusion method (Paper V)	272	109	381

All isolates were stored in Brain Heart Infusion (BHI) broth with 15% glycerine at -80 °C and were cultivated at 37 ºC anaerobically on Brucella blood agar supplemented with haemin (0.005 g/l) and vitamin K1 (0.01 g/l) (Becton Dickinson, Heidelberg, Germany) in an anaerobic cabinet (Concept 400; Ruskinn Technology Ltd., Bridgend, UK) using a gas composition of 85% N_2, 10% H_2 and 5% CO_2 for 48 h.

The MIC values of the nine antimicrobial agents (ampicillin, cefoxitin, clindamycin, amoxicillin/clavulanic acid, piperacillin/tazobactam, tigecycline, imipenem/cilastatin, metronidazole and moxifloxacin) were determined previously by the agar dilution technique, as recommended by the CLSI and published by Nagy *et al*. in 2011 [http://clsi.org/]. Reference strains used during the studies looking for resistance and toxin genes in *B. fragilis* and related species are listed in the **Table 3**.

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

3.2.1. Antibiotic resistance determination by disc diffusion

The inoculum from the 24 h primary plates of the isolates (**Table 2.**) involved in the evaluation of the applicability of the disc distribution test for the antibiotic resistance determination was prepared in physiological saline to reach McFarland 1. The 15-15-15-minute rule of EUCAST

Table 3. Reference strains used during this study for the detection of known antibiotic resistance genes and the enterotoxigenic gene

Gene	Control strain
cepA	*B. fragilis* 638R
cfiA	*B. fragilis* TAL3636
cfxA	*B. vulgatus* CLA341
bft1	*B. fragilis* VPI 13783
bft3	*B. fragilis* GAI 96478
nimA	*B. fragilis* 638R (plP*417*)
nimB	*B. fragilis* BF8
nimC	*B. thetaiotaomicron* BT13
nimD	*B. fragilis* 638R (plP*421*)
nimE	*B. fragilis* 388
ermB	*C. difficile* 630
ermF	*B. fragilis* BF8
ermG	*B. thetaiotaomicron* 4001 (pGERM)
linA	*B. fragilis* TR23
mefA	*B. thetaiotaomicron* 4001 (pGERM)
msrSA	*B. thetaiotaomicron* 4001 (pGERM)
tetM	*C. difficile* 630
tetQ	*B. vulgatus* CLA341
tetX	*B. fragilis* BF8
tetX1	*B. fragilis* BM13
tet36	*Escherichia coli* DH5α (pGW140.1)
bexA	*E. coli* AG102AX (pBRT20)

was used. This means that the inoculum suspension is used within 15 min after preparation, the disks are placed on the inoculated plates within 15 min and then the pates are placed in the correct incubation atmosphere (in our case in the anaerobic environment) within another 15 min. Nine antibiotics were tested during the disc diffusion measurements on Brucella blood agar supplemented with haemin and vitamin K1 (Becton Dickinson, Heidelberg, Germany). The antibiotic discs were as follows: amoxicillin/clavulanic acid (20/10 μg/disc), piperacillin/tazobactam (30/6 μg/disc), cefoxitin (30 μg/disc) imipenem/cilastatin (10 μg/disc),

meropenem (10 μg/disc), clindamycin (10 μg/disc), tigecycline (15 μg/disc), metronidazole (5 μg/disc), moxifloxacin (5 μg/disc). All discs were obtained from BioRad (Marnesla-Coquette, France) except metronidazole and clindamycin, which were purchased from Oxoid (Basingstoke, UK). The plates were incubated at 37 ºC in an anaerobic atmosphere for 24 h in GasPak™ EZ Standard Incubation Container (Becton Dickinson, Heidelberg, Germany). Zone diameters were read at 100% inhibition. All the measurements were carried out with the naked eye using a caliper. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used as reference strains for the evaluation of the disc diffusion method.

3.2.2. MIC determination by the E-test

For some strains the disk diffusion results showed very major error (susceptible by disc diffusion and resistant by the previous MIC determination). In all these cases the experiments were repeated. The E-test strips (bioMerieux, Marcyl'Etoile, France) were applied on the same plate as the corresponding antibiotic discs and because of this the same culture conditions were provided.

B. fragilis ATCC 25285 was used as the reference strain for all MIC measurements, using the different antibiotic discs and the corresponding E-test on the same plate. No inter-laboratory or inter-personal reproducibility evaluation was carried out. All the measurements were done by the PhD candidate.

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

To detect the various antibiotic resistance genes and *bft* gene the bacterial cells from the surface of 24 h anaerobic agar plates were suspended in 100 μl distilled water in 1.5 ml Eppendorf tubes, and incubated at 100 ºC for 10 min. The supernatants of the centrifuged suspensions (2 min, 14,000 rpm) were used as template DNA and stored at -20 ºC until use. Primers suitable for providing products in RT-PCR experiments, using the known nucleotide sequences of the genes, were designed by the Primer3 software (http://frodo.wi.mit.edu/). Each reaction mixture contained a 5 μl 2x PCR “mastermix” (iQ, Bio-Rad or Brilliant II, Stratagene), 0.7 μM (35 pmoles) of each primer, 1 μl template DNA, 0.5 μl EvaGreen (Biotium) DNA-binding fluorescent dye (for the iQ “mastermix”) dye and sterile water up to 10 μl final volumes in plastic PCR plates. Amplification was performed in MxPro3000 (Stratagene, USA) or StepOne (Life-Technologies) Real-Time PCR instruments. The amplification and the melting curves were observed at a wavelength of 415 nm

required for the SYBR Green and the EVA Green dyes. The initial denaturation by the amplification cycles was 10 min (iQ) or 5 min (Brilliant II). The PCR conditions for the various genes, primer sequences and PCR parameters are given in **Table 4**. Positive reactions were identified by the starting amplification cycle, melting curves showing the correct melting temperatures, and in rare cases where it was required to compare the size of the products with those of the positive controls in 1.2% agarose gel electrophoresis. Nucleotide sequencing of the *tetX1* (*B. fragilis* BM13) and *linA* (*B. fragilis* TR23) was carried out as described previously [Brisson-Noël and Courvalin, 1986; Whittle *et al*., 2001], and their sequences were compared to the reference sequences (*linAn2* AF251288 and *tetX1* AJ311171) by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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

The *bft* alleles of the *bft* gene positive strains obtained by the RT-PCR were determined by PCR-RFLP. Using the BTT1 (CATGTTCTAATGAAGCTGATTC) and BTT2 (ATCGCCATCTGCTGTTTCCC) primers the entire *bft* genes were amplified in end-point PCRs (95 ºC 10 min 1x; 95 ºC 30 sec, 62 ºC 1 min, 72 ºC 1 min, 35x). The PCR products were purified with the HighPure PCR Cleanup Kit (Roche Diagnostics GmbH, Mannheim, Germany) after agarose gel electrophoresis and the pure products were digested with *Mbo*I restriction enzyme. The final products were analyzed by 1.5% agarose gel electrophoresis in TBE buffer, using 0.5 µg/ml ethidium bromide and UV visualization. The PCR products using the BTT1 and BTT2 primers were used in PCR RFLP and the expected sizes of the digested fragments were as follows: 839 and 310 bp (*bft1*); 575, 453 and 111 bp (*bft2*); 839, 189 and 111 bp (*bft3*), respectively. Reference strains used for this method are shown in **Table 3**.

Table 4. Oligonucleotide primers and the parameters used for the detection of antibiotic resistance genes and *bft* gene with Real-Time PCR analysis

Gene	Primer 5'→3'[a]	PCR cycles
cepA	TTTCTGCTATGTCCTGCCT ATCTTTCACGAAGACGGC	95°C 15 sec, 56 °C 30 sec, 72 °C 1 min, 35x
cfiA	AATCGAAGGATGGGGTATGG CGGTCAGTGAATCGGTGAAT	94 °C 15 sec, 59 °C 1 min, 72 °C 30 sec, 40x
cfxA	TGACTGGCCCTGAATAATCT ACAAAAGATAGCGCAAATCC	95 °C 15 sec, 55 °C 1 min, 72 °C 30 sec, 35x
nim	ATGTTCAGAGAAATGCGGCGTAAGTG GCTTCCTCGCCTGTCACGTGCTC	94 °C 15 sec, 62 °C 30 sec, 72 °C 30 sec, 35x
ermB	GCGGAATGCTTTCATCCTAA GCGTGTTTCATTGCTTGATG	95°C 15 sec, 59°C 30 sec, 72 °C 30 sec, 35x
ermF	TAGATATTGGGGCAGGCAAG GGAAATTGCGGAACTGCAAA	95 °C 15 sec, 58 °C 1 min, 72 °C 30 sec, 35x
ermG	ATAGGTGCAGGGAAAGGTCA TGGATTGTGGCTAGGAAATGT	95°C 15 sec, 59°C 30 sec, 72 °C 30 sec, 35x
linA	CTGGGGAGTGGATGTCTTGT AGTTGGCTTGTTTGGAAGTG	95°C 15 sec, 60°C 30 sec, 72 °C 30 sec, 32x
mefA	ATACCCCAGCACTCAATTCG CAATCACAGCACCCAATACG	95°C 15 sec, 59°C 30 sec, 72 °C 30 sec, 35x
msrSA	GGGAACTGAAAGATGGCAAA TACGAGCCTGTTTTCGCTTT	95°C 15 sec, 60°C 30 sec, 72 °C 30 sec, 32x
tetM	ATCCTTTCTGGGCTTCCATT TCCGTCACATTCCAACCATA	95°C 15 sec, 59°C 30 sec, 72 °C 30 sec, 35x
tetQ	ATCGGTATCAATGAGTTGTT GACTGATTCTGGAGGAAGTA	95 °C 15 sec, 50 °C 1 min, 72 °C 30 sec, 35x
tetX	TTAGCCTTACCAATGGGTGT CAAATCTGCTGTTTCATTCG	95°C 15 sec, 55°C 30 sec, 72 °C 30 sec, 35x
tetX1	TCAGGACAAGAAGCAATGAA TATTTCGGGGTTGTCAAACT	95°C 15 sec, 50°C 1 min, 72 °C 30 sec, 35x
tet36	TTTCTGGCAGAGGTAGAACG TTAATTCCTTGCCTTCAACG	95°C 15 sec, 57°C 30 sec, 72 °C 30 sec, 35x
bexA	TAGTGGTTGCTGCGATTCTG TCAGCGTCTTGGTCTGTGTC	95°C 15 sec, 60°C 30 sec, 72 °C 30 sec, 32x
bft	CGAACTCGGTTTATGCAGTT GGATACATCAGCTGGGTTGT	95 °C 15 sec, 56 °C 1 min, 72 °C 30 sec, 35x

[a] Forward and then the reverse primer sequences

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

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

3.6. Detection of plasmids

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

3.7. Statistical evaluation

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

4. RESULTS

4.1. Evaluation of disc diffusion method for antibiotic susceptibility testing of *B. fragilis* group isolates (Paper V)

In this study 381 *B. fragilis* group clinical isolates were involved (see **Table 2**.) from the same collection of isolates used for the antibiotic resistance surveillance during the Europe-wide study. The strains were collected in different countries in Europe as clinical isolates and the antibiotic susceptibility of the strains was previously tested by agar dilution method and the MICs were determined [Nagy *et al.,* 2011]. As reference strains *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used, recommended by CLSI for the antibiotic resistance determination by agar dilution. The distribution of the inhibition zone diameters obtained by the disc diffusion method and MICs for the eight antibiotics where MIC data were available is illustrated in the histograms as used by EUCAST [http://www.eucast.org/] for demonstrating the relationship between MICs and zone diameters to prove the applicability of the disc diffusion method for the antibiotic resistance determination of some less slow growing anaerobic isolates such as *B. fragilis* group strains (**Fig. 1 A-H**).

In the case of imipenem only four isolates were resistant (MIC >8 μg/ml) and two isolates showed intermediate susceptibility (MIC 4-8 μg/ml). For each MIC value the inhibition zones varied from 0 to 13 mm, with 90% of the values within 8 mm. However, the resistant isolates were clearly separated from the susceptible strains: inhibition zone diameter for the resistant strains was ≤ 20 mm and for the susceptible strains ≥ 29 mm, respectively (**Fig. 1. A.**).

The same was true for metronidazole where only 2 isolates were found, which were resistant according to the EUCAST breakpoints (**Table 5.**), with an MIC 8 μg/ml. All but one of the susceptible isolates had an inhibition zone ≥ 24 mm. The inhibition zones for the different MIC values varied between 0 and 14 mm and 90% of the values were within 6 mm (**Fig 1.B.**).

According to the MIC data no amoxicillin/clavulanic acid resistant isolate was among the strains tested. A very large distribution of the inhibition zones of the strains with the same MICs was seen (8-14 mm), however intermediate resistant strains (MIC 8 μg/ml) had an inhibition zone ≤22 mm with some overlap with susceptible isolates (**Fig.1.C.**)

Fig. 1. Distribution of inhibition zone diameters and MICs for 381 *B. fragilis* group isolates with different antibiotics. **A**: imipenem (10 μg/disc), **B**: metronidazole (5 μg/disc), **C**: amoxicillin/clavulanic acid (20/10 μg/disc), **D**: piperacillin/tazobactam (30/6 μg/disc), **E**: clindamycin (10 μg/disc), **F**: cefoxitin (30 μg/disc), **G**: moxifloxacin (5 μg/disc), **H**: tigecycline (15 μg/disc). Each isolate is shown in the zone diameter histogram in a color representing its MIC value.

Fig. 1.A.

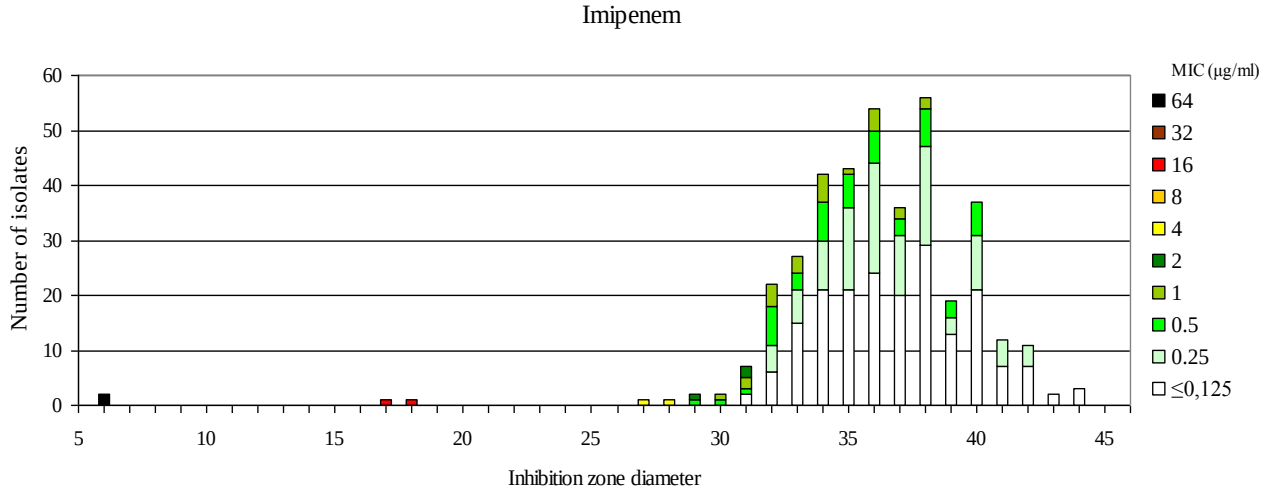


Fig. 1.B.

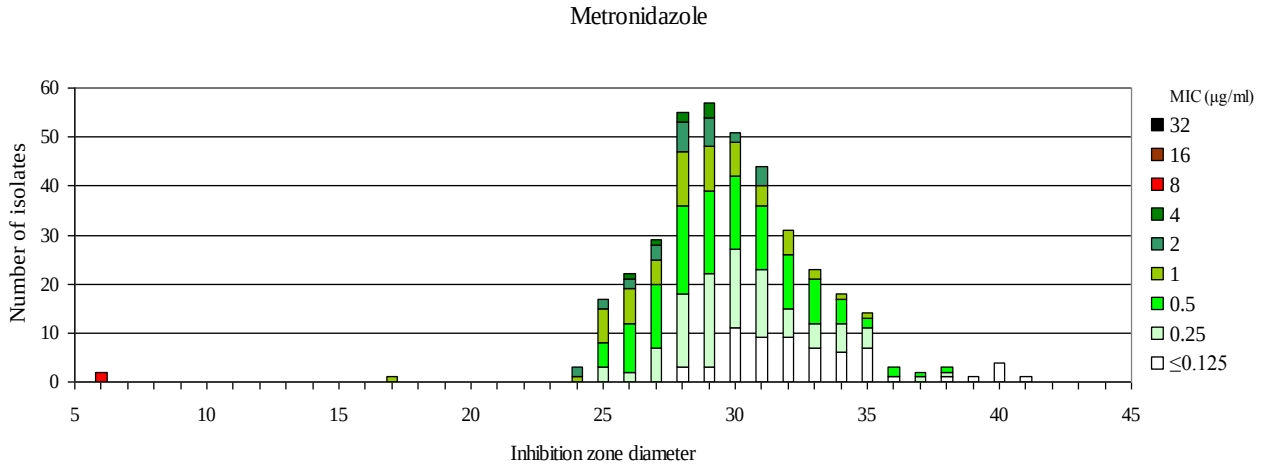


Fig. 1.C.

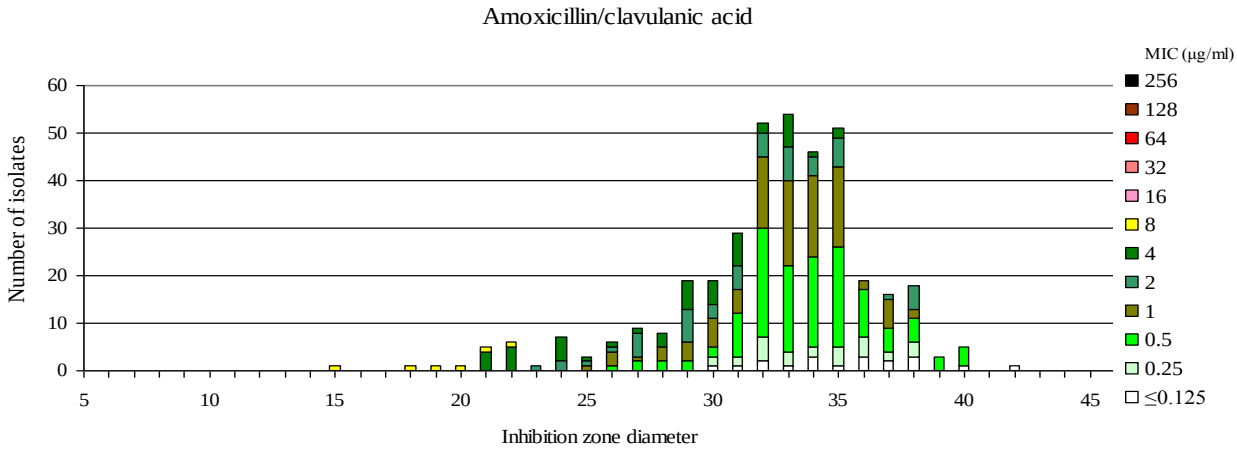


Fig. 1.D.

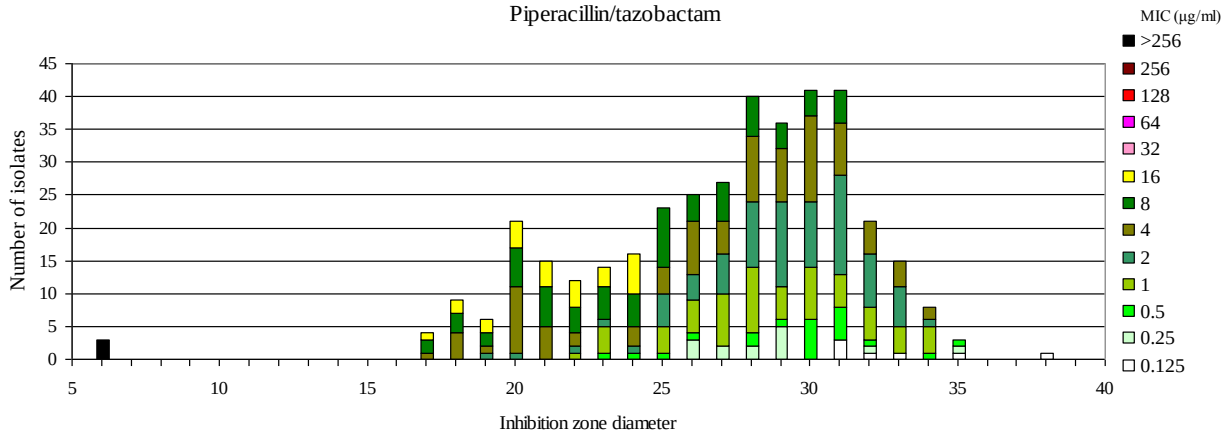


Fig. 1.E.

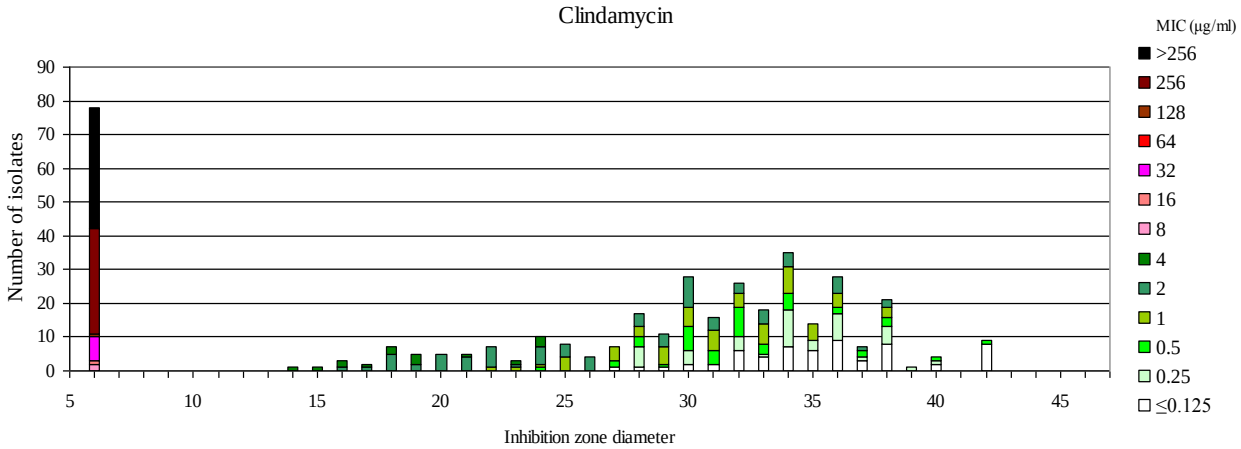


Fig. 1.F.

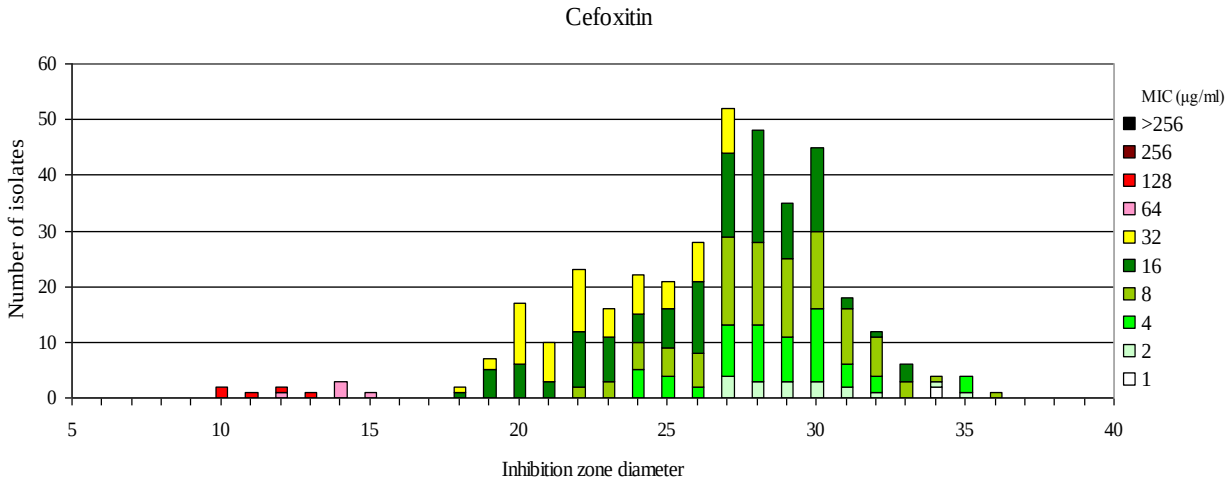


Fig. 1.G.

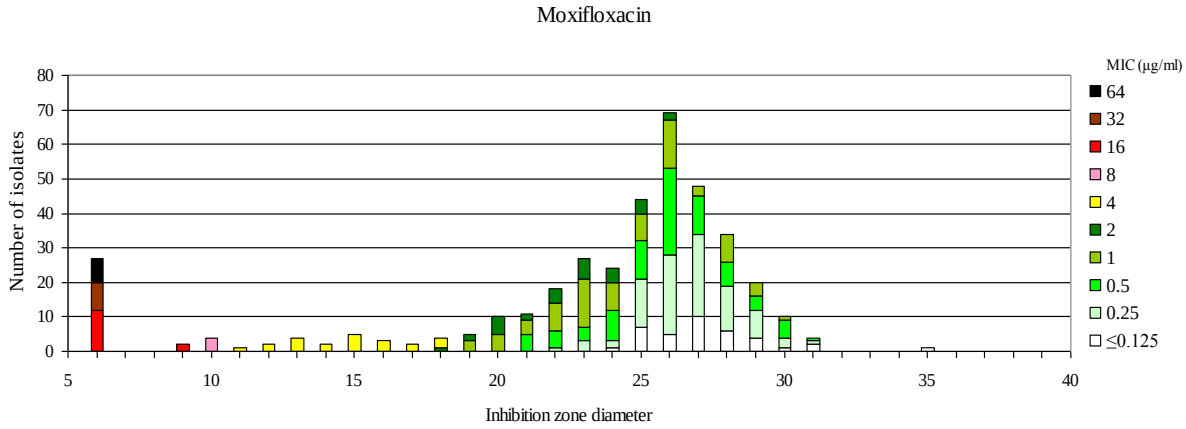
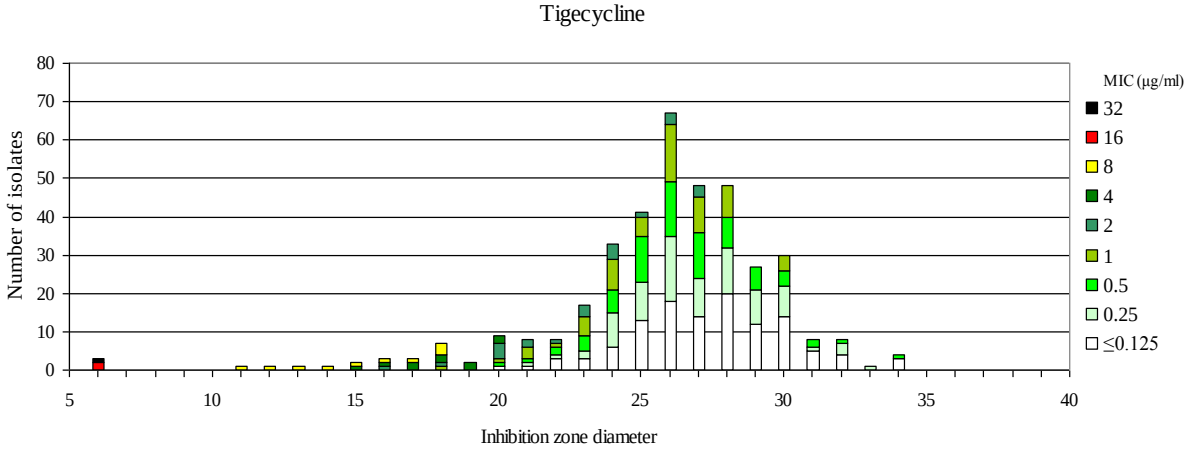


Fig. 1.H.



In the case of piperacillin/tazobactam, three resistant strains were found clearly separated from the intermediate and susceptible population by an inhibition zone ≤16 mm, however intermediate and susceptible isolates overlapped by the disc diffusion method. According to these data, isolates with an inhibition zone ≤24 mm and >16 mm should be tested by the E-test to determine MICs and differentiate fully susceptible from intermediate susceptible isolates. The distribution of inhibition zone diameters for each MIC values varied between 0 and 17 mm (**Fig 1.D.**).

For testing clindamycin susceptibility of the *Bacteroides* strains by disc diffusion the 10 μg/disc was obtained instead of the 2 μg/disc, used for the antibiotic susceptibility testing of aerobic bacteria. This clearly separated the resistant population with an inhibition zone ≤13 mm. The results showed that the inhibition zone diameters for the clindamycin susceptible strains stretched out between 14 and 42 mm, however the very susceptible (MIC ≤0.125 μg/ml) isolates had a larger inhibition zone diameter in average than those having higher MICs. The distribution of inhibition zone diameters for each MIC values varied between 0 and 23 mm (**Fig.1.E.**).

In the case of cefoxitin only CLSI breakpoints are available. According to those the susceptible isolates had inhibition zone diameters between 18 mm and 36 mm. The isolates with intermediate MICs (32 μg/ml) had inhibition zone between 18 mm and 27 mm, but this range overlapped with the fully susceptible isolates (MIC ≤16 μg/ml). However, the resistant population (MIC >32 μg/ml) was separated from the susceptible isolates with an inhibition zone ≤15 mm. The zone diameters varied for the different MICs between 0 and 15 mm (**Fig 1.F.**).

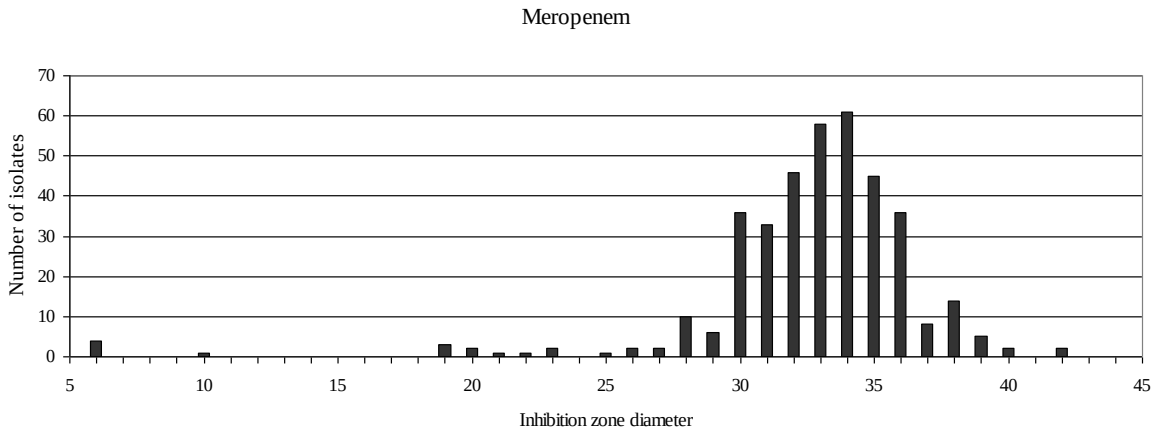
For moxifloxacin only CLSI breakpoints are available (**Table 5.**) (**Fig.1.G.**). The disk diffusion test clearly separated the susceptible isolates with an inhibition zone ≥19 mm. Only few isolates were found in the intermediate range with an inhibition zone between 11 mm and 18 mm. All the resistant isolates (MIC >4 μg/ml) had a zone diameter ≤10 mm. The zone diameters varied for the different MICs between 0 and 11 mm with 95% within 6 mm.

For tigecycline (**Fig. 1.H.**) no MIC breakpoints are available in the EUCAST or CLSI documents, accordingly only comparison of the MICs and the zone diameters was possible. Among the strains tested only three were found which could be considered fully resistant and had no inhibition zone at all with MICs ≥32 μg/ml. All the strains which had MICs ≤4 μg/ml could be separated with a zone diameter ≥20 mm. For each MIC value the inhibition zones varied from 0 to 11 mm, with 90% of the values within 6 mm.

For meropenem we did not have MIC data from the previous European surveillance study. Most of the strains had a zone diameter ≥28 mm, which separated the few isolates, which can be considered intermediate susceptible or resistant to carbapenems (**Fig. 2.**). If we compared the

distribution of the zone diameters of imipenem and meropenem more isolates not belonging to the wild type strains (being fully susceptible) could be detected by the meropenem disc (**Fig. 2.**) This is in agreement with some other studies where meropenem could better distinguish between carbapenem susceptible and intermediate susceptible isolates [Toprak *et al*., 2012].

Fig. 2. Distribution of inhibition zone diameters of meropenem (10 μg) disc for 381 *B. fragilis* group isolates.



According to these data, with one exception (cefoxitin), we could suggest tentative zone diameter breakpoints using the disc diffusion method for susceptible isolates of *B. fragilis* group strains based on the MIC breakpoints set by EUCAST and for some antibiotics by CLSI (**Table 5.**).

Table 5. EUCAST MIC breakpoints for the antibiotics tested and the suggested susceptibility zone diameter breakpoints

Antimicrobial agents	EUCAST clinical MIC breakpoints (μg/mL)		Suggested zone diameter breakpoints (mm)
	≤S	>R	≤S
Imipenem	2	8	29
Metronidazole	4	4	24
Amoxicillin/clavulanic acid	4	8	15
Piperacillin/tazobactam	8	16	25
Clindamycin	4	4	25
Cefoxitin[a]	16	32	?
Moxifloxacin[a]	2	4	19

[a] Only CLSI breakpoints are available.
?: means that no zone diameter sensitivity breakpoint decision was possible.

4.1.1. Re-testing of isolates with discrepant results obtained by the disc diffusion method and the agar dilution MIC determination

In those cases, where discrepant results such as major errors (when isolates were susceptible by agar dilution method, but resistant by disk diffusion), or very major errors (susceptible with the disc diffusion method and resistant according to the agar dilution method) were found, the MIC determination was repeated by E-test and disc diffusion zone diameters were also determined on the same agar plate. Data of these repeated measurements were taken in consideration during the final data processing shown in **section 4.1**. The MICs measured by E-test confirmed disc diffusion result in most of the cases**.** (**Fig. 3. Fig. 4.**)

Fig. 3. Parallel measurements of the zone diameter and the MIC by E-test in case of HR54 *B. fragilis*

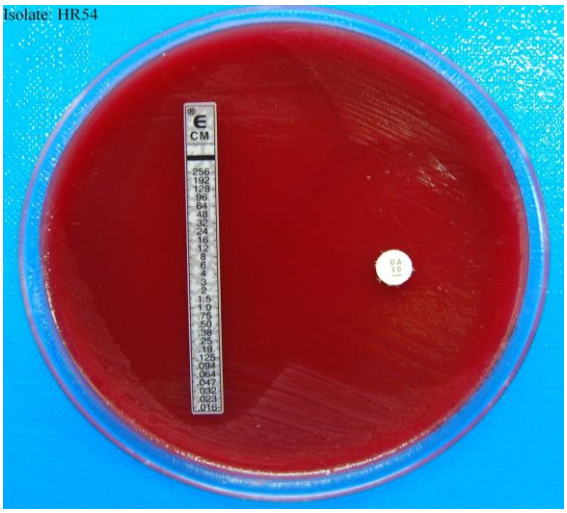
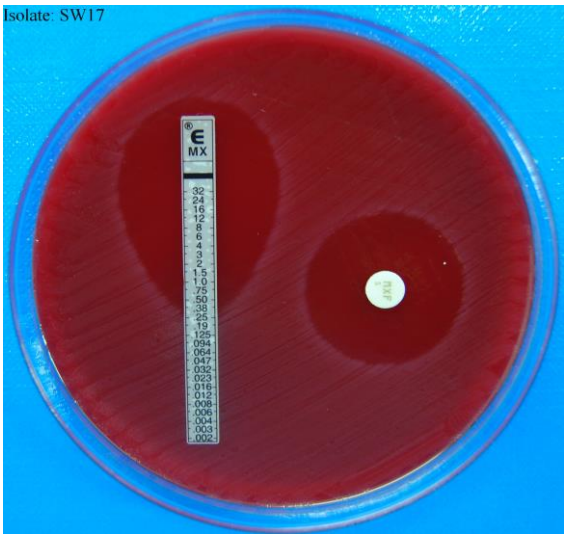


Fig. 4. Parallel measurements of the zone diameter and the MIC by E-test in case of SW17 *B. fragilis*



The evaluation of the results of the repeated disk diffusion measurements gave also an opportunity to check the reproducibility of the measurements of the inhibition zones. Out of the 113 measurements (different isolates and different antibiotic discs), 88.5% of the repeated measured inhibition zone diameters were within 0-3 mm (**Fig. 5**). We also evaluated the reproducibility of the zone diameter for the reference strain *B. fragilis* ATCC 25285 used throughout the experiments. The standard deviations of the zone diameters for the *B. fragilis* ATCC 25285 based on 15 parallel measurements were between 0.5 and 2.2 mm for the different antibiotics (**Table 6.**).

Fig. 5. Zone diameter differences between repeated measurements (altogether 113)

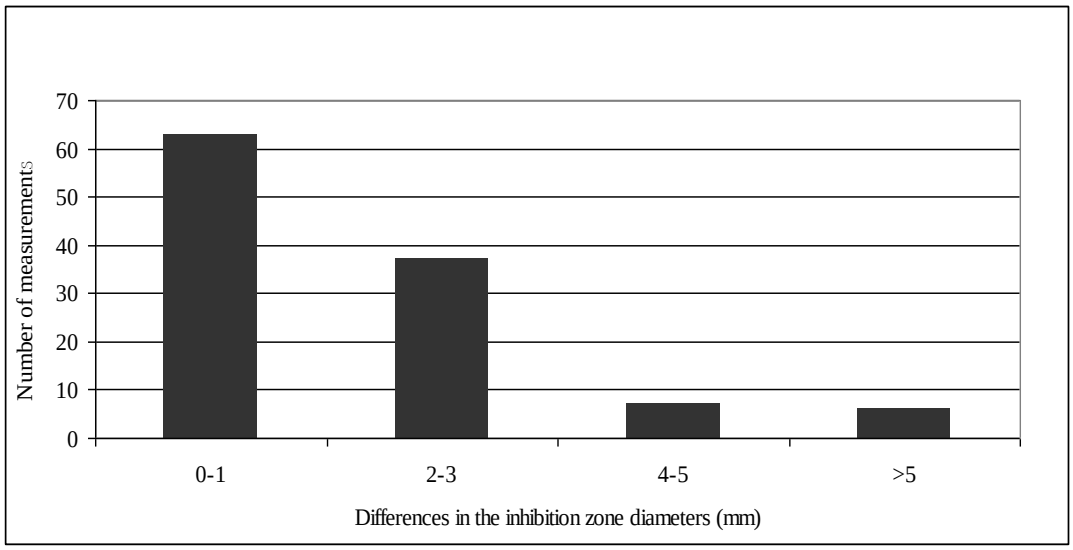


Table 6. Standard deviation of the zone diameters of different antibiotic discs based on 15 parallel measurements

B. fragilis ATCC 25285	Antibiotic discs									
	AMC (20/10	TZP (30/6 µg)	CFX (30 µg)	IMI (10 µg)	MER (10 µg)	DA (10	TET (30 µg)	TIGE (15 µg)	MTZ (5 µg)	MXF (5 µg)
Range of zone diameter (mm)	34.0	32.0	30.0	37.0	35.0	30.0	33.0	28.0	31.0	31.0
Average of 15 control measurements during the study	34.0	30.7	28.8	36.6	33.6	30.3	30.8	27.7	29.2	29.7
SD	1.6	1.1	2.0	2.5	2.2	0.5	2.1	1.5	1.4	1.0

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

In this part of the study we were interested in the prevalence of the two most important resistance genes among a large cohort of the *Bacteroides* isolates originated from all over Europe. Out of the 640 *Bacteroides* fragilis groups strains 43 (6.7%) harbored the *cfiA* gene and 3 (0.5%) was *nim* gene positive (**Table 7.**). All the *cfiA* positive isolates belonged to *B. fragilis* giving an 8.8% positivity of the 486 isolates belonging to this species. Out of the 43 *cfiA* positive *B. fragilis* strains 33 proved to be imipenem sensitive during the MIC determination with MIC <4 µg/ml (data not shown), which shows the wider carriage rate of this resistance gene among the *B. fragilis*

clinical isolates than the expression of the carbapenem resistance.

Table 7. Prevalence of *cfiA* , *nim* and *bft* genes among the 640 *Bacteroides* strains collected for the antibiotic resistance surveillance from Europe

Resistance gene	*Bacteroides* (n=640)	*B. fragilis* (n=486)	NFB (n=154)
cfiA	43 (6.7)	43 (8.8)	0 (0.0)
nim	3 (0.5)	2 (0.4)	1 (0.6)
bft	68 (10.6)	68 (14.0)	0 (0.0)
bft1 allel	51	51	0
bft2 allel	15	15	0
bft3 allel	2	2	0
bft + *cfiA*	4	4	0

From the 640 *Bacteroides* isolates examined 22 had an imipenem MICs ≥4 μg/ml (non susceptible) and out of these 7 isolates had an MIC ≥16 μg/ml (belonging to the resistant category). Out of the 22 imipenem non-susceptible isolates (MICs ≥4 μg/ml) 10 harbored the *cfiA* gene (**Table 8.**). Of the 10 *B. fragilis* strains with elevated imipenem MICs (4–8 μg/ml) four (40.0%) were *cfiA*-positive, while 6 (85.7%) of the 7 imipenem-resistant (MIC ≥16 μg/ml) *B. fragilis* isolates were *cfiA*-positive. No non-fragilis *Bacteroides* strains were resistant to imipenem. A *cfiA*-negative, but imipenem-resistant *B. fragilis* isolate was identified in this study (*B. fragilis* FI37) with a possible other resistance mechanisms than *cfiA*-mediated carbapenemase activity (**Table 8.**).

Among the strains with MIC 4 μg/ml to imipenem and harboring the *cfiA* gene (*B. fragilis* IT15) an IS element has been shown upstream of the *cfiA* gene (IS4351) by PCR mapping. Among the *cfiA*-positive and imipenem-resistant strains (MIC ≥16 μg/ml) four harbored IS elements upstream of the resistance gene (**Table 8.**). The remaining two *cfiA*-positive isolates that were imipenem-resistant, but without activating IS elements upstream of *cfiA* displayed a heterogeneous resistance phenotype shown by the imipenem E-test (**Fig. 6**). The types of *cfiA*-activating IS elements were IS1187 (n=2), IS614B (n=1), and a novel IS element (n=ISBf11; GenBank accession no. GQ449386) was also described for *B. fragilis* NLH3 that had 77% homology compared with IS614B. *B. fragilis* IT15 harbored IS*4351* upstream of the *cfiA* gene, but its imipenem MIC was lower (4 μg/ml) (intermediate resistant). Two highly imipenem-resistant strains (*B. fragilis* HU61 and FR41) were also genetically "silent", their *cfiA* genes not being activated by IS elements. This phenomenon can be explained by activation of the *cfiA* genes till yet

unidentified mechanism that boosts the carbapenemase activity of the strains.

Fig. 6. Heterogeneous imipenem-resistant phenotype of *Bacteroides fragilis* FR41 detected by E-test. The first (0.25 μg/mL) and second (4 μg/mL) inhibition zones are marked by thin and thicker arrows, respectively.

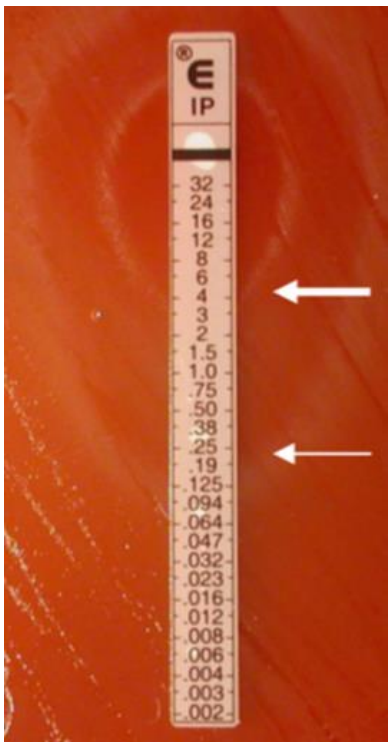


Table 8. Analysis of the imipenem resistance mechanism of strains with elevated imipenem minimum inhibitory concentrations (MICs) (≥4 μg/mL)

Strain	Imipenem MIC (μg/mL)	*cfiA*	Upstream region	Mechanism
B. fragilis SW42	4	-	-	Other[a]
B. fragilis SW46	4	-	-	Other
B. fragilis SW83	4	-	-	Other
B. fragilis TR38	4	-	-	Other
B. fragilis HU25	4	-	-	Other
B. fragilis FI63	4	-	-	Other
B. eggerthii GR67	4	-	-	Other
B. thetaiotaomicron BEM28	4	-	-	Other
Parabacteroides merdae GR70	4	-	-	Other
B. fragilis DE14	4	+	280 bp[b]	„Silent" with increased MIC
B. fragilis HU51	4	+	280 bp[b]	„Silent" with increased MIC
B. fragilis IT15	4	+	IS*4351*	IS-activated
B. stercoris HU59	8	-	-	Other
B. thetaiotamicron BEA22	8	-	-	Other
B. fragilis HU92	8	+	280 bp[b]	„Silent" with increased MIC
B. fragilis TR27	16	+	IS*1187*	IS-activated
B. fragilis TR31	16	+	IS*1187*	IS-activated
B. fragilis HU61	32	+	280 bp[b]	Heteroresistant
B. fragilis NLH3	>32	+	IS*Bf11*	IS-activated
B. fragilis FR41	>32	+	280 bp[b]	Heteroresistant
B. fragilis FI87	>32	+	IS*614B*	IS-activated
B. fragilis FI37	>32	-	-	Other

[a] The effects are not caused by *cfiA*.

[b] The 280-bp PCR fragment displays no insertions upstream of *cfiA*.

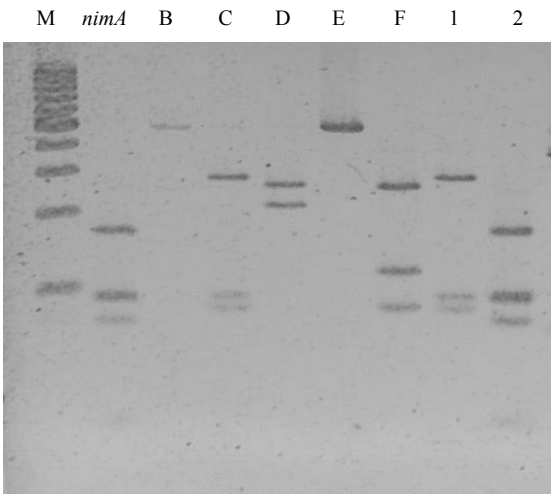
Of the 640 *Bacteroides* strains, 21 had reduced susceptibility to metronidazole (MIC ≥ 4 μg/ml) and only 3 (*B. fragilis* IT724 and IT797 and *B. thetaiotaomicron* HU66) harbored *nim* genes

(**Table 7.** and **Table 9.**), with the following metronidazole MICs 0.125 μg/ml (*B. fragilis* IT797), 1 μg/ml (*B. fragilis* IT724) and 256 μg/ml (*B. thetaiotaomicron* HU66) (**Table 9.**). An examination of the *nim*-mediated resistance mechanisms revealed that *B. fragilis* IT797 and IT724 harbored chromosomal *nimA* and *nimC* genes, respectively. By contrast, the *nimE* gene of *B. thetaiotaomicron* HU66 was located on an 8.3 kb (pBF388c-like) plasmid described earlier [Sóki *et al*., 2006] and was activated by ISBf6 (**Fig. 7.**). No *nim*-specific plasmids were detected in the two other strains. Furthermore, *B. fragilis* IT797 harbored IS1168 and IS1170, but these elements could not be mapped before the *nimA* gene by PCR mapping. The *nim*-negative but metronidazole-resistant *Bacteroides* strains found in the current study may have other resistance mechanisms (reduced uptake, nitroreductase and pyruvate–ferredoxin oxidoreductase activities, increased lactate dehydrogenase activity, or mutations that alter the carbohydrate utilization affecting the redox state) which shortcut the detrimental cellular effects of this drug.

Table 9. List of *Bacteroides* strains with metronidazole MIC ≥4 µg/mL and/or *nim* gene positivity

Strain	Metronidazole (µg/mL)	*nim* (location)	IS
B. fragilis IT797	<0.125	*nimA* (chromosomal)	IS*1168*, IS*1170*
B. fragilis IT724	1	*nimC* (chromosomal)	-
B. fragilis NE12/1932	4	-	-
B. fragilis NE25/2010	4	-	-
B. fragilis NE37/1782	4	-	-
B. vulgatus HU30252	4	-	-
B. fragilis HU83915	4	-	-
B. fragilis HU85277	4	-	-
B. fragilis HU25341	4	-	-
B. fragilis HU22317	4	-	-
B. stercoris HU20720	4	-	-
B. fragilis HU20895	4	-	-
B. fragilis BM16	4	-	-
B. fragilis FI53112	4	-	-
B. fragilis FI53128	4	-	-
B. fragilis FI53157	4	-	-
B. fragilis FR58	4	-	-
B. fragilis FR34	4	-	-
B. thetaiotaomicron FR40	4	-	-
B. fragilis SP4	8	-	-
B. fragilis FR41	16	-	-
B. fragilis FI53168	32	-	-
B. thetaiotaomicron HU66	256	*nimE* (pBF388c-like)	IS*bf6*

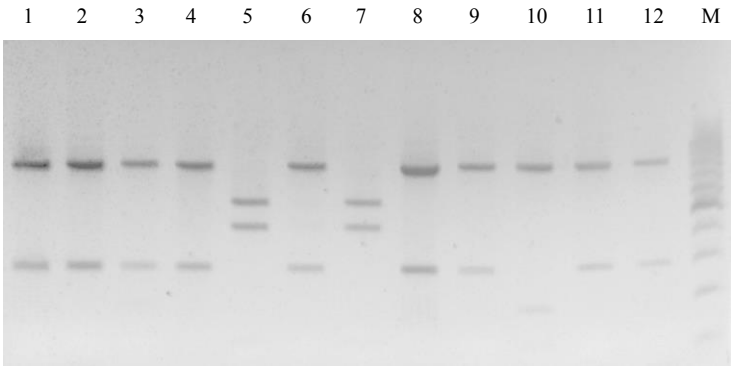
Fig. 7. PCR-RFLP patterns of the *nimA* to *nimF* genes cleaved with restriction enzyme *Hpa*II. 1. *B. fragilis* IT724 harbored *nimC* gene. 2. *B. fragilis* IT797 harbored *nimA* gene. M: molecular marker bands (GeneRuler 100 bp DNA Ladder Plus, Fermentas, Vilnius, Lithuania).



4.3. Investigation of the prevalence of the *bft* gene among the isolates and determination of the *bft* alleles (Paper II)

Among the 640 *Bacteroides* strains studied, 68 were *bft*-positive (10.6%) (**Table 7.**). All *bft* positive strains belonged to *B. fragilis* providing a 14.0% of prevalence among these isolates. During the PCR RFLP analysis we found that 51 (75.0%) carried the *bft1* allele, 15 (22.1%) carried the *bft2* allele and 2 (2.9%) carried the *bft3* allele (**Table 7.**, **Fig. 8.**). To explore the roles of these alleles in non-intestinal pathogenesis of *B. fragilis*, we checked the distribution of the three *bft* types in isolates originating from different clinical samples, especially among blood culture isolates. The overall *bft* prevalence and the prevalence of the *bft1-3* alleles among the strains examined in this study were not significantly elevated among the blood culture isolates (9.2% vs. 7.3%), but the number of isolates obtained from blood cultures was rather low (n=5), which could be the cause of the non-significant test result (data not shown in detail).

Fig. 8. PCR-RFLP analysis for characterization of *bft* genes cleaved with restriction enzyme *Mbo*I. 1-4. *B. fragilis* 657, 45416, 20895 and TR11 (*bft1*); 5. IT722 (*bft2*); 6. IT749 (*bft1*); 7. GR582 (*bft2*); 8-9. GR456 and GR434 (*bft1*); 10. GR513 (*bft3*); 11-12. GR510 and FI53138 (*bft1*). M: molecular marker bands (GeneRuler 100 bp DNA Ladder Plus, Fermentas, Vilnius, Lithuania).



4.3.1. Analysis of the *cfiA*-*bft* doubly positive *B. fragilis* strains **(Paper II)**

Of the 486 *B. fragilis* strains, 43 were *cfiA*-positive (**Table 7.**). Surprisingly, of the 68 *bft*-positive *B. fragilis* strains, 4 were also *cfiA*-positive (**Table 7.** and **Table 10.**). Comparing the prevalence of the *bft* genes among the *cfiA*-negative (64 out of 443 strains) (14.4%) and *cfiA*-positive (4 out of 43 strains) (9.3%) strains by using Fischer's exact test did not reveal a

statistically significant difference, which means a basically equal distribution of the *bft* genes among *cfiA*-positive and -negative *B. fragilis* strains (data not shown). The four *bft-cfiA* doubly positive strains were isolated in four different European countries (Belgium, Hungary, Italy and Turkey) and we have data about three of them originating from sever infection (**Table 10.**).

Table 10. Characteristics of the *bft-cfiA* doubly positive *B. fragilis* strains

Characteristics	*B. fragilis*				
	R19811 (UK[a]) blood	TR6 (Turkey[b]) intra-abdominal abscess	BEB15 (Belgium[b]) wound	IT9 (Italy[b]) not known	HU92 (Hungary[b]) periproctal abscess
cfiA	+	+	+	+	+
Imipenem MICs (μg/mL)	≥256	0.5	2	2	8
cfiA upstream	IS614B[a]	IS-	IS-	IS-	IS-
bft gene allel	*bft* 1	*bft* 1	*bft* 1	*bft* 1	*bft* 1

[a] Isolate investigated and published by Terhes *et al*., 2007

[b] Isolates investigated in this study

The *bft1* allel was found in all 4 doubly positive isolates found in this study and also in a 5th isolate originating from the UK and investigated earlier in our laboratory [Terhes *et al.,* 2007]. The presence of the *bft* gene together with a "silent" *cfiA* gene was found in all four isolates from this study and also with an "expressed" *cfiA* gene providing antibiotic resistance for imipenem beside the enterotoxic activity of the strain from the previous study.

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

In this study, a more detailed molecular analysis was performed to learn more about the incidence and distribution of the different resistance genes already described to be present among *B. fragilis* group strains. Out of the 640 strains which were tested for the presence of the *cfiA* and *nim* gene, we chose 161 (128 *B. fragilis* and 33 non-fragilis *Bacteroides*) strains (see **Table 2.**) in order to detect the occurrence of the following further genes: *cepA, cfxA, ermB, ermF, ermG, linA, mefA, msrSA, tetM, tetQ, tetX, tetX1*, *tet36* and *bexA*. The selection criteria of the strains were intended to represent the whole collection, taking into account how many strains were collected by the different countries originally and in particular one country (Hungary). The distribution of the genes among the strains by countries is shown in **Table 11**. The most prevalent resistance genes were *tetQ* (80.1%), *cepA* (70.2%), *ermF* (24.2%) and *linA* (21.7%) with no significant difference among the different European countries. No *nim, tetM* and *tet36* gene was detected among these

161 isolates (**Table 11.**).

We also compared the prevalence of the tested genes among the *B. fragilis* isolates (128) and those which belonged to different other species of the genus *Bacteroides* (33). (**Table 12.**). *CfiA, ermB, ermG* and *msrSA* were only detected in *B. fragilis* isolates, however no significant other differences were observed in the prevalence of the other genes among *B. fragilis* and non-fragilis isolates.

Table 12. Prevalence of the investigated antibiotic resistance genes among the *B. fragilis* and non-fragilis *Bacteroides* strains

Bacteroides fragilis group strains	Number of strains with resistance gene[a]												
	cepA	*cfxA*	*cfiA*	*ermB*	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*	*tetQ*	*tetX*	*tetX1*	*bexA*
B. fragilis (n=128)	101	19	12	1	29	9	28	17	9	101	13	7	6
Non-fragilis *Bacteroides* (n=33)	12	10	0	0	10	0	7	3	0	28	3	1	6
Altogether:	113	29	12	1	39	9	35	20	9	129	16	8	12

[a] Non of these isolates harboured the *nim*, *tetM* and *tet36* gene

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

All the *B. fragilis* strains (128) were resistant to ampicillin (MIC ≥ 2 μg/ml) and 101 of them (78.9%) harbored the *cepA* gene. Among the 33 non-fragilis *Bacteroides* strains which were also resistant to ampicillin, only 12 of them (36.4%), carried the *cepA* gene (**Table 13.**).

The *cepA* gene distributed with significantly different frequencies among *B. fragilis* and non-fragilis *Bacteroides* strains ($p < 0.001$). The presence of the *cepA* gene did not correlate with the ampicillin MIC values of the tested strains (**Fig. 9.**); rather, it occurred among all ampicillin MIC ranges (from 2 to 256 μg/ml). Out of the 11 cefoxitin-resistant *B. fragilis* strains, 3 of them (27.3%) harbored the *cfxA* gene and out of the 9 cefoxitin resistant non-fragilis *Bacteroides* strains just 1 (11.1%) harbored the *cfxA* gene (**Table 13.**).

Table 11. Distribution of tested resistance genes among the 161 *B. fragilis* group isolates taken from different European countries

Countries (number of *B. fragilis* group strains)	Number of the strains harboring the resistance genes (%)[a]												
	cepA	*cfxA*	*cfiA*	*ermB*	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*	*tetQ*	*tetX*	*tetX1*	*bexA*
Belgium (n=23)	13 (56.5)	11 (47.8)	3 (13.0)	0 (0.0)	7 (30.4)	1 (4.3)	7 (30.4)	4 (17.4)	1 (4.3)	21 (91.3)	3 (13.0)	1 (4.3)	1 (4.3)
Croatia (n=20)	14 (70.0)	3 (15.0)	0 (0.0)	0 (0.0)	5 (25.0)	0 (0.0)	3 (15.0)	0 (0.0)	0 (0.0)	16 (80.0)	1 (5.0)	1 (5.0)	2 (10.0)
Czech Republic (n=15)	12 (80.0)	1 (6.7)	1 (6.7)	0 (0.0)	2 (13.3)	0 (0.0)	3 (20.0)	3 (20.0)	0 (0.0)	9 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)
France (n=4)	4 (100.0)	0 (0.0)	0 (0.0)	1 (25.0)	2 (50.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)
Germany (n=18)	15 (83.3)	2 (11.1)	2 (11.1)	0 (0.0)	3 (16.7)	0 (0.0)	3 (16.7)	2 (11.1)	0 (0.0)	16 (88.9)	1 (5.6)	0 (0.0)	1 (5.6)
Greece (n=10)	5 (50.0)	0 (0.0)	1 (10.0)	0 (0.0)	3 (30.0)	0 (0.0)	2 (20.0)	0 (0.0)	0 (0.0)	8 (80.0)	2 (20.0)	0 (0.0)	0 (0.0)
Hungary (n=48)	37 (77.1)	7 (14.6)	4 (8.3)	0 (0.0)	7 (14.6)	3 (6.4)	12 (25.0)	4 (8.3)	3 (6.3)	37 (77.1)	4 (8.3)	1 (2.1)	4 (8.3)
Sweden (n=8)	3 (37.5)	0 (0.0)	1 (12.5)	0 (0,0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (62.5)	0 (0.0)	0 (0.0)	2 (25.0)
Turkey (n=16)	10 (62.5)	5 (31.3)	1 (6.3)	0 (0.0)	9 (56.3)	5 (31.3)	4 (25.0)	7 (43.8)	5 (31.3)	14 (87.5)	4 (25.0)	4 (25.0)	2 (12.5)
Altogether: 161	113 (70.2)	29 (18.0)	12 (7.5)	1 (0.6)	39 (24.2)	9 (5.6)	35 (21.7)	20 (12.4)	9 (5.6)	129 (80.1)	16 (9.9)	7 (4.3)	12 (7.5)

[a] Non of these isolates harboured the *nim, tetM* and *tet36* gene

The relationship between the cefoxitin MIC values of the strains and the carrying of the *cfxA* gene is depicted in **Fig. 10.** Interestingly, among the strains with high MIC values (64 μg/ml), the *cfxA* gene was often absent. Also, in contrast to *cepA*, this gene was more common among non-fragilis *Bacteroides* strains (p=0.039).

Table 13. Distribution of the *cepA*, *cfxA*, and *cfiA* genes among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of the genes with the *Bacteroides* strains resistance to ampicillin, cefoxitin and imipenem

Bacteroides strains		Number of strains with resistance genes (%)		
		cepA	*cfxA*	*cfiA*
B. fragilis (n=128)		101 (78.9)[b]	19 (14.8)[c]	12 (9.4)
Non-fragilis *Bacteroides* (n=33)		12 (36.4)[b]	10 (30.3)[c]	0 (0.0)
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistant strains with resistance genes (%)		
		cepA	*cfxA*	*cfiA*
B. fragilis (n=128)	Ampicillin	101 (78.9)	-	-
B. fragilis (n=11)	Cefoxitin	-	3 (27.3)	-
B. fragilis (n=1)	Imipenem	-	-	1 (100.0)
Non-fragilis *Bacteroides* (n=33)	Ampicillin	12 (36.4)	-	-
Non-fragilis *Bacteroides* (n=9)	Cefoxitin	-	1 (11.1)	-
Non-fragilis *Bacteroides* (n=0)	Imipenem	-	-	0 (0.0)

Ampicillin ≥ 2 μg/mL Cefoxitin ≥ 64 μg/mL Imipenem ≥ 16 μg/mL.

[a] Resistance breakpoints according to CLSI (MIC μg/mL)

[b] The difference is statistically significant ($p < 0.001$) between values marked by the superscript b letters.

[c] The difference is statistically significant ($p = 0.039$) between values marked by the superscript c letters.

Fig. 9. The distribution of *cepA*-positive and *cepA*-negative *Bacteroides* strains in terms of ampicillin MIC values.

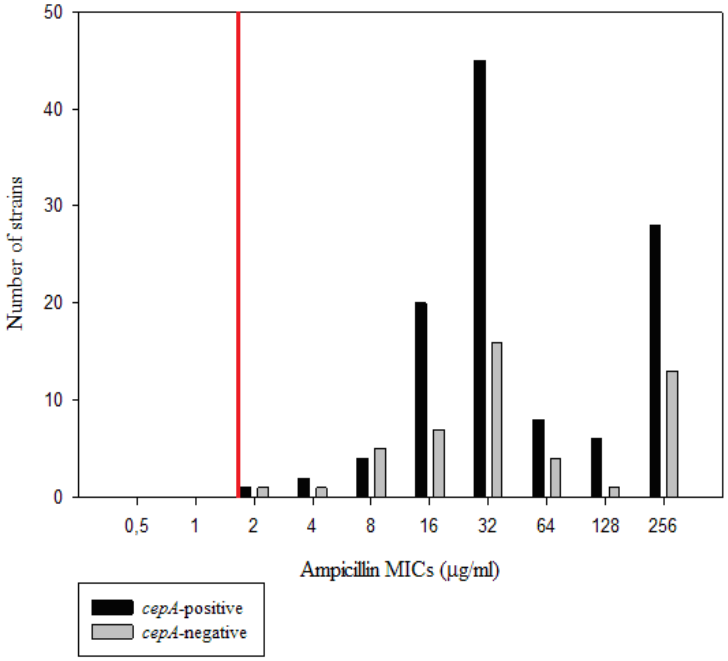
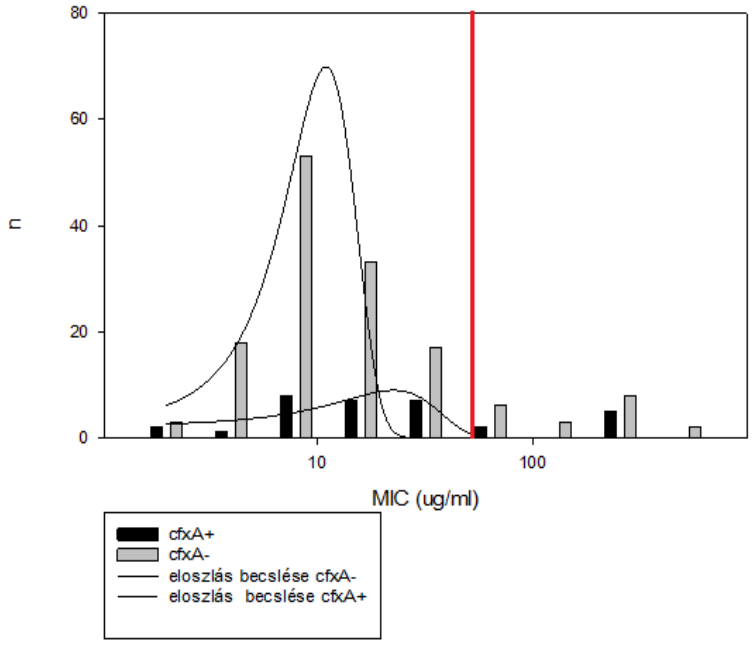


Fig. 10. The distribution of *cfxA*-positive and *cfxA*-negative *Bacteroides* strains in terms of the cefoxitin MIC values.



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

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

Table 14. Distribution of the *ermB*, *ermF*, *ermG*, *linA*, *mefA* and *msrSA* genes among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of the genes with the *Bacteroides* strains resistance to clindamycin

Bacteroides strains		Number of strains with resistance genes (%)					
		ermB	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*
Bacteroides fragilis (n=128)		1 (0.8)	29 (22.7)	9 (7.0)[b]	28 (21.9)	17 (13.3)	9 (7.0)
Non-fragilis *Bacteroides* (n=33)		0 (0.0)	10 (30.3)	0 (0.0)[b]	7 (21.2)	3 (9.1)	0 (0.0)
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistance strains with resistance genes (%)					
		ermB	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*
Bacteroides fragilis (n=31)	Clindamycine	1 (3.2)	23 (74.2)	9 (29.0)[c]	9 (29.0)[d]	11 (35.5)[e]	9 (29.0)
Non-fragilis *Bacteroides* (n=9)		0 (0.0)	7 (77.8)	0 (0.0)[c]	5 (55.6)[d]	0 (0.0)[e]	0 (0)

[a] Resistance breakpoint for clindamycin: ≥8 µg/mL according to CLSI

[b,c] The differences between the prevalences are notable, but not statistically significant (p= 0.117 and p= 0.09, respectively) between values marked by the superscript b and c letters.

[d] The observed difference is not statistically significant (p= 0.234) between values marked by the superscript d letters, probably because of the low number of test strains.

[e] The difference is statistically significant (p= 0.043) between values marked by the superscript e letters.

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

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

The prevalence of the *tetQ*, *tetX* and *tetX1* genes among 161 *Bacteroides* strains were 129 (80.1%), 16 (9.9%) and 8 (5.0%), respectively (**Table 15.**). There were no *tetM*-positive or *tet36*-positive strain among the *Bacteroides* strains tested. Only 3 (1.9%) *B. fragilis* strains were resistant to tigecycline (MIC 16 µg/ml) and all of them carried the *tetQ* gene. Moreover, the *tetM*, *tetX*, *tetX1* and *tet36* genes were not present in any of the tigecycline-resistant *Bacteroides* strains (data not shown).

Table 15. Distribution of the *tetM*, *tetQ*, *tetX*, *tetX1* and *tet36* genes among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of the genes with the *Bacteroides* strains resistance to tigecycline

Bacteroides strains		Number of strains with resistance genes (%)				
		tetM	*tetQ*	*tetX*	*tetX1*	*tet36*
Bacteroides fragilis (n=128)		0 (0.0)	101 (78.9)	13 (10.2)	7 (5.5)	0 (0.0)
Non-fragilis *Bacteroides* (n=33)		0 (0.0)	28 (84.8)	3 (9.1)	1 (3.0)	0 (0.0)
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistance strains with resistance genes (%)				
		tetM	*tetQ*	*tetX*	*tetX1*	*tet36*
Bacteroides fragilis (n=3)	Tigecycline	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Non-fragilis *Bacteroides* (n=0)		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Resistance breakpoint for tigecycline: ≥ 16 μg/mL according to CLSI.

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

The *bexA* gene, which was considered to be responsible for the moxifloxacin resistance, was present in 12 (7.5%) of the 161 *Bacteroides* isolates tested. These consisted of 6 (4.7%) *B. fragilis* strains and 6 (18.2%) non-fragilis *Bacteroides* strains (**Table 16.**). This difference is statistically significant (p=0.024). There were no *bexA*-positive strains among the 18 moxifloxacin-resistant *B. fragilis* isolates, and of the 6 moxifloxacin resistant non-fragilis *Bacteroides*, only one (16.7%) harbored the *bexA* gene.

Table 16. Distribution of the *bexA* gene among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of their presence with the resistance of the *Bacteroides* strains to moxifloxacin

Bacteroides strains		Number of strains with resistance genes (%)
		bexA
Bacteroides fragilis (n=128)		6 (4.7)[b]
Non-fragilis *Bacteroides* (n=33)		6 (18.2)[b]
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistance strains with resistance genes (%)
		bexA
Bacteroides fragilis (n=18)	moxifloxacin	0 (0.0)[c]
Non-fragilis *Bacteroides* (n=6)		1 (16.7)[c]

[a] Resistance breakpoint for moxifloxacin: ≥8 µg/mL according to CLSI.

[b] The difference between the prevalences are statistically significant (p= 0.024) between values marked by the superscript b letters.

[c] The observed difference is not statistically significant (p= 0.250) between values marked by the superscript c letters, probably because of the low number of test strains.

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

Romania was not part of the *Bacteroides* antibiotic resistance surveillance carried out in 2008-2009, because of this 53 isolates (36 *B. fragilis* and 17 non-fragilis *Bacteroides*) (see **Table 2.**) were tested and the data were compared with the data of the Europe-wide study. Despite of the fact that only small number of strains were tested, all the antibiotic resistance tendencies observed during the Europe-wide study could be detected (**Table 17.**).

Table 17. Antibiotic susceptibilities of the 53 Romanian *Bacteroides* strains

Antibiotic agent	MIC (µg/mL)			Resistant strains (%)		
	MIC range	MIC_{50}	MIC_{90}	Romanian *B. fragilis*	Romanian non-fragilis *Bacteroides*	European *B. Fragilis* group strains[a]
Ampicillin	1 – >256	32	>256	97.3	94.1	98.2
Amoxicillin/clavulanic acid	1 – 64	2	16	13.5	11.8	10.4
Cefoxitin	2 – 128	16	64	2.7	41.2	17.2
Clindamycin	0.032 – >256	4	8	2.8	23.5	32.4
Imipenem	0.064 – 2	0.25	1	0.0	0.0	0.8
Metronidazole	0.125 – 1	0.25	0.5	0.0	0.0	0.5
Moxifloxacin	0.5 – >32	1	>32	13.5	17.6	13.6
Piperacillin/tazobactam	1 – 32	4	16	2.7	11.8	10.3
Tetracycline	<0.5 – 256	32	64	73.0	82.4	-
Tigecycline	0.032 – 8	0.25	0.5	0.0	0.0	1.7

[a] Nagy *et al*., 2011.

Resistance to ampicillin was 96.3%, and 54.7 % of the resistant strains carried the *cepA* gene**.** Among the 8 cefoxitin-resistant isolates only 2 harbored the resistance gene, the *cfxA*. Resistance to amoxicillin/clavulanic acid was 13.0%. 73.0% of the *B. fragilis* strains and 82.4% of the non-fragilis *Bacteroides* were resistant to tetracycline. The resistant strains 78.0% (32) harbored the *tetQ* gene. No imipenem- and metronidazole-resistant isolates were found, however 3 *B. fragilis* strains harbored the *cfiA* gene silently. 11 (20.4%) isolates were resistant to clindamycin. The occurrence of the relevant resistance gene of clindamycin of all the tested 55 strains was the following: 4 *ermF*, 3 *linA*, 2 *msrSA* and 1 *ermB*, respectively. 100% of the *ermF*-positiv strains (4) were clindamycin-resistant. Resistance to moxifloxacin was 13.5% of the *B. fragilis* isolates and 17.6% of the non-fragilis *Bacteroides* isolates. 9 *bexA*-postive isolates were detected, all of these strains were susceptible of moxifloxacin.

The prevalence of the tested antibiotic resistance genes among *B. fragilis* and non-fragilis *Bacteroides* isolates originated from Romania are listed in **Table 18**.

Table 18. Prevalence of the investigated antibiotic resistance genes among the 53 *Bacteroides* isolates collected in Romania

B. fragilis group strains	Number of strains with reistance gene [a]											
	cepA	*cfxA*	*cfiA*	*ermB*	*ermF*	*linA*	*msrSA*	*nim*	*tetM*	*tetQ*	*tetX*	*bexA*
B. fragilis strains (n=36)	32	5	3	0	1	1	0	1	0	25	1	0
non-fragilis *Bacteroides* strains (n=17)	1	2	0	1	3	2	2	0	1	14	1	9
Altogether	33	7	3	1	4	3	2	1	1	39	2	9

[a] Non of these isolates harbored the *ermG, mefA, tetX1* and *tet36* gene

5. DISCUSSION

B. fragilis group strains are the most frequently isolated anaerobic pathogens in clinical microbiological laboratories. They may cause severe intra-abdominal, postoperative wound infections, special skin and soft-tissue infections and also sepsis. Most commonly they are found in mixed infections, but they are also important constituents of the normal colonic microflora. Infections involving anaerobic bacteria, including *B. fragilis* group isolates, are usually treated empirically, based on surveillance reports about the susceptibility patterns of these pathogens. Consequently, it is very important to regularly carry out these antimicrobial sensitivity surveillances to have a real picture about the current resistance levels of these microorganisms.

Antibiotic susceptibility testing of anaerobic bacteria, especially *B. fragilis* group strains is becoming more and more important also for the clinical microbiological laboratories, as the susceptibility of them have become increasingly unpredictable during the past decades. Resistance development to the most active antibiotics such as carbapenems, piperacillin/tazobactam, amoxicillin/clavulanic acid and metronidazole has been reported during nation-wide or European studies [Behra-Miellet *et al*., 2003; Snydman *et al*., 2010; Nagy *et al*., 2011; Wybo *et al*., 2014]. Studies showed that the ampicillin resistance is almost 100% among the *B. fragilis* group strains. After ampicillin, the highest resistance rates were found to clindamycin, 28.5–60% for the different *Bacteroides* species in Europe. In Argentina, the resistance rate to clindamycin was ~40% among *B. fragilis* group strains in 2012 [Fernández-Canigia *et al*., 2012] and 38% of the tested strains were resistant to clindamycin during a US surveillance study as well. [Snydman *et al*., 2011]. Furthermore, increasing resistance to carbapenems and penicillins in combination with β-lactamase inhibitors are also registered. The resistance rates to amoxicillin/clavulanic acid and to piperacillin/tazobactam among the *Bacteroides spp.* are around 10% in a large European study [Nagy *et al*., 2011]. Resistance to cefoxitin is also increasing both in Europe (17.2%) and in the USA (11.2%) [Nagy *et al*., 2011; Snydman *et al*., 2011] The level of imipenem resistance has not changed dramatically during the past 20 years (0%, <1% and 1.2%), although the percentage of isolates with reduced susceptibilities (MIC ≥4 μg/ml) has increased continuously (0.3%, 1.6% and 2.7%) [Nagy *et al*., 2011], however, the number of the imipenem-resistant isolates in Asia, for example South Korea is also quite high (4%) [Lee *et al*., 2010]. The rate of metronidazole resistance or reduced susceptibility in *Bacteroides spp*. is in general low (1-4%).

Several papers report about cases caused by broadly multidrug-resistant *B. fragilis* isolates, and also about the development of resistance *in vitro* or *in vivo* in the presence of antibiotics [Turner *et al*., 1995; Wareham *et al*., 2005; Katsandri *et al*., 2006; Kalapila *et al*., 2013]. These factors emphasize the need for antimicrobial susceptibility testing of anaerobes not only during periodical surveillances in different geographical areas, but also during the everyday routine laboratory testing.

5. 1. Evaluation of disc diffusion method for antibiotic susceptibility testing of *B. fragilis* group isolates (Paper V)

Among the methods which are standardized today for antibiotic susceptibility testing of anaerobic bacteria the quantitative E-test is used in most laboratories in Europe, but for many laboratories in low income countries it is too expensive to be used on the regular bases. Other methods are not for routine use (such as agar dilution method) or are influenced by many factors (such as broth micro-dilution or spiral gradient endpoint system) to get reproducible results. For rapid growing anaerobes (*Bacteroides spp*. or most of the clostridia) disc diffusion method could be a solution. Earlier several groups used this method for evaluation the activities of anti-anaerobic drugs, however standardization of culture circumstances (media, inoculum and incubation) were not fully solved [Wilkins *et al*., 1972; Oitmaa and Benn, 1981; Calliham and Nolte, 1985; Horn *et al*., 1987; Barry, 1990].

In 1966, Bauer *et al*. developed the disc diffusion test for aerobic bacteria (the Bauer-Kirby method). In 1972, Wilkins *et al*. used a single-disc diffusion technique for determination antibiotic susceptibility of anaerobic bacteria (such as *Clostridium*, *Peptostreptococcus* and *Bacteroides*) to seven antibiotics. The method was standardized by correlation of zone diameters with minimal inhibitory concentrations determined by broth macro dilution method. In 1975, Kwok *et al*. determined the susceptibility of 55 slow-growing anaerobes to eight antibiotics by agar dilution and disk diffusion tests. Correlation between minimal inhibitory concentration and inhibition zone diameters was generally good. Horn *et al.* (1987) modified the disc diffusion test, developed by Bauer *et al*. (1966) in order to permit testing of anaerobes that grow well after overnight incubation. They tested 225 isolates of the *B. fragilis* group to six antibiotics and determined the antibiotic susceptibility by a disk diffusion test on Wilkins-Chalgreen agar

and by the standard agar dilution method. Zone diameters were measured after 24 h. They found generally good correlation between the MICs and diameters of inhibition zone for cefoxitin, clindamycin, moxalactam and ticarcillin. However, in the Wadsworth Manual (2002) used by most routine laboratories in the USA and Europe the semi-quantitative disc diffusion method is not recommended for antibiotic susceptibility testing of anaerobic bacteria on the routine bases mostly because the great differences in growth dynamic of different anaerobic species, but even in the case of "rapid" growing species the results could be influenced by the proper anaerobic environment, selection of media and preparation of inoculum.

The disc diffusion method for susceptibility testing of facultative and aerobic bacteria is accepted world-wide. Recently EUCAST [http://www.eucast.org/], started to harmonize the disc diffusion method for antimicrobial susceptibility testing and classification of resistance for anaerobic bacteria as well, first of all as emergence of reduced susceptibility towards metronidazole and vancomycin was noticed among *C. difficile* strains, which may very much influence the treatment of this rapidly spreading, nosocomial diarrhoea cases and further more because of the growing number of reports about infections caused by multidrug-resistant *Bacteroides* isolates. The EUCAST methodology of the disk diffusion method was started to be evaluated for *C. difficile* by Erikstrup *et al.* (2012). The media, which was used in that study is the Brucella Blood Agar supplemented with hemin and vitamin K1 (BBA) [Justesen *et al.,* 2012] recommended for antimicrobial susceptibility testing of anaerobic bacteria by the E-test. They also tested the effects of the changes in CO_2 levels and temperature on the inhibition zone diameters. They found an excellent agreement between inhibition zone diameters by disk diffusion and MICs determined by E-test for *C. difficile* strains.

Extensive study was carried out to find the proper media for the disc diffusion testing of rapid growing *Bacteroides* isolates as well [Justesen *et al*., 2011a; 2011b] and the first evaluation was presented during the ECCMID 2013 [Luu *et al.,* 2013]. In our study testing a large number of *B. fragilis* group isolates (381) we used standardized inoculum preparation, the supplemented Brucella blood agar obtained from the same supplier (BD, Heidelberg, Germany) through out of the study and a fully controlled anaerobic incubation for exactly 24 hours. A large selection of *B. fragilis* and other *Bacteroides* spp were tested against 9 antibiotics with differences in the MICs against these antibiotics. The only drawback of the study was that MIC data were collected several years earlier for the strains, which were stored in -80 ^{o}C afterwards, till we used

them in our study. This may result differences in the resistance levels of some isolates. However, discrepant results observed, in most cases very major errors (namely: susceptible by disc diffusion and resistant by the previous MIC determination) were cleared up by repeated measurements on the same plate with the E-test and the disc of the same antibiotic. Reproducibility of the disc diffusion measurements for the control strains were carried out in two different locations. In Odense during testing the composition of the broth for the inoculum and the effect of the pre-reduction of the plates no significant differences were observed in the zone diameters for meropenem, metronidazole and piperacillin/tazobactam for the control strains such as *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 (**Paper V**). In Szeged, the standard deviation of zone diameters during the parallel measurements of the 9 antibiotics for the *B. fragilis* ATCC 25285 were between 0.5 and 2.2 mm (**Table 6.**), independent of the time points of the measurement, or the lots of the media.

With one exception (cefoxitin) we could suggest tentative zone diameter breakpoints for susceptible *B. fragilis* group strains (**Table 5.**). We found a good agreement between the inhibition zone diameters and the MICs for imipenem, metronidazole, moxifloxacin and tigecycline similar to the previous study testing 104 *B. fragilis* group isolates [Luu *et al.,* 2013]. The inhibition zone diameters of meropenem also separated clearly the isolates, which can be considered wild-type isolates. In case of amoxicillin/clavulanic acid and piperacillin/tazobactam intermediate and susceptible isolates according to the MIC determination overlap during the zone diameter determination. Isolates with an inhibition zone <23 mm for amoxicillin/clavulanic acid and <25 mm for piperacillin/tazobactam should be retested by a MIC determination method to differentiate susceptible and intermediate isolates. Different from previous studies [Oitmaa and Benn, 1981; Luu *et al.,* 2013] instead of the 2 µg, the 10 µg clindamycin disc was used during this study and a clear separation of resistant and susceptible population of *B. fragilis* group strains was observed in this way. However, we have not evaluated its applicability for the detection of the inducible clindamycin resistance. In the case of cefoxitin only the resistant population could be separated with an inhibition zone <17 mm, intermediate and susceptible isolates overlap using the disc diffusion method. Accordingly, no zone diameter breakpoint could be suggested for susceptibility to cefoxitin according to our data.

5.2. Investigations on the prevalence of *bft* and a large series of known antibiotic

resistance genes among *B. fragilis* group clinical isolates (Paper I, II, III and IV)

Beside the phenotypic investigation of antibiotic resistance of anaerobic bacteria (including *B. fragilis* group strains), it is highly important to collect data about the prevalence of the resistance genes among the clinical isolates. Several earlier studies have shown that a wide range of resistance genes can be found on the chromosome or on specific plasmids of *Bacteroides* strains, which may be silent or be responsible for high level resistance to different antibiotics [Edwards, 1997; Avelar *et al*., 2003; Löfmark *et al*., 2005; Boente *et al*., 2010; Bartha *et al*. 2011]. Our aim was not only to look for the presence of the specific resistance genes in different consortia of *B. fragilis* group isolates, but also to evaluate their connection with the phenotypic presence of resistance to different antibiotics. Genetic methods, compared to conventional phenotypic susceptibility methods, have the potential to provide a more rapid and reliable assessment of antimicrobial resistance. a) Genetic susceptibility testing methods can be performed directly with clinical specimens obviating the need for isolation of the organism by culture. b) These methods assess the genotype of the organism, whereas conventional susceptibility techniques asses the phenotype or expression of the genotype under artificial or laboratory conditions. c) In some cases, genotypes may be discerned long before phenotypes can be determined due to the slow growth of the organism. d) Some organisms cannot be cultured or are not easily cultured such as anaerobic bacteria and so only genotypes can be determined in these cases [Cockerill, 1999]. Not only the prevalence of the resistance genes among our isolates were compared with earlier literature data, but also with phenotypic resistance data. For a subset of strains we also investigated the coexistence of the known virulence gene of *B. fragilis*, the *bft* gene with different important resistance genes.

5.2.1. The prevalence of the *cfiA* and *nim* genes among 640 clinical *Bacteroides* isolates originated from Europe and investigation of the IS elements activating these genes **(Paper I, IV)**

According to different studies the prevalence of the imipenem resistance among *Bacteroides* strains is about 1%, however the presence of the *cfiA* gene responsible for most of the carbapenem resistance in *B. fragilis* isolates is much higher. In different earlier studies the prevalence of the *cfiA* gene tested by hybridization or by PCR was

between 1.9 % and 6.9 % in different countries. [Yamazoe *et al*., 1999; Edwards *et al*., 1999; Sóki *et al*., 2000], however much higher percentage of *cfiA* positivity (27%) was also reported among 66 clinical *Bacteroides* isolates from Turkey (all belonging to *B. fragilis*) [Toprak *et al.,* 2012]. In our experiments 8.8% of the tested 486 *B. fragilis* clinical isolates were positive for the *cfiA* gene by PCR, however most of these isolates had an imipenem MIC <4 μg/ml (33 of 43 isolates) showing that a high number of the isolates are carrying this resistance gene in a "silent" form.

Previous studies have shown that the imipenem resistance is not transferable, but resistant clones can be selected after a one-step mutation at a frequency of ca. 10^{-7} from suitable strains usually with an imipenem MIC=1 μg/ml, carrying a "silent" carbapenemase gene [Podglajen *et al*., 1992]. This mutation means an IS element insertion into the promoter region of a *cfiA* gene and this results a high-level expression of the carbapenemase gene and the phenotypic appearance of resistance to carbapenems [Podglajen *et al*., 1994]. The *cfiA* gene without IS element called "silent" *cfiA* gene, and these strains can express only very low amounts of β-lactamase. When we compared the presence of the *cfiA* gene among those *Bacteroides* isolates, which proved to be non-susceptible for imipenem (MIC ≥4 μg/ml) only *B. fragilis* isolates (10) harboured the *cfiA* gene, but only 5 had also an IS element (IS4351, IS1187, ISBf11 or IS614B) before the gene. In most cases this was enough to have an imipenem MIC 16 or >32 μg/ml (**Table 8.**). Only 3 "silent" *cfiA* gene harbouring strains were detected among the 36 *B. fragilis* isolates originating from the Romanian study, showing a very similar prevalence (8.3%) of this gene to that of found for the large collection of the European isolates. Our present study, similarly to some earlier studies from our institute [Sóki *et al*., 2004a; 2004b] detected some elevated imipenemase activities in "silent" *cfiA*-positive strains that could account for non-susceptibility to imipenem in the case of these isolates.

Compared to the prevalence of the *cfiA* gene (6.7%) and the *nim* gene (0.5%) among the 640 tested *Bacteroides* strains we have found great difference despite of the fact that the number of the metronidazole non-susceptible isolates (MIC >4 μg/ml) was similar to that of imipenem non-susceptible isolates (No. 22). Out of the three *nim* positive isolates (2 *B. fragilis* and 1 non-fragilis *Bacteroides*) only one had a metronidazole MIC 256 μg/ml with an IS*Bf6* before the gene which was located on a plasmid, while the two "silent" *nim* genes were located on the chromosome. The *nim*-negative strains with elevated MIC to metronidazole found in the current study may have other resistance mechanisms (such as reduced uptake, nitroreductase and pyruvate-

ferredoxin oxidoreductase activities, increased lactate dehydrogenase activity or mutations that alter the carbohydrate utilisation affecting the redox state) which shortcut the detrimental cellular effects of metronidazole [Rasmussen *et al*., 1993; Narikawa *et al*., 1991; Diniz *et al*., 2004; Patel *et al*., 2009]. All these mechanisms were not tested during this study.

Similar to the large cohort of the European isolates in our Romanian study out of the 53 *Bacteroides* isolates there was also only one (1.8%) *B. fragilis* isolate with a "silent" *nimB* gene present on the chromosome. Two moderately metronidazole resistant *B. fragilis* isolates were reported from our Institute in 2001 [Nagy *et al*., 2001] with the presence of *nim* gene chromosomally. The gene was activated by the IS1168/IS1186 element in the case of these strains. Opposite to our present study no *nim* gene was found among 242 metronidazole susceptible *Bacteroides* isolates at that time. Several studies from Europe and other parts of the world proved the presence of the different *nim* genes (*nim* A-I) among *Bacteroides* strains causing resistance to metronidazole and possible failure of treatment of severe infections [Jamal *et al*., 2004; Gal and Brazier, 2004; Sóki *et al*., 2006; Trinh and Reysset, 1996; Stubbs *et al*., 2000]

5.2.2. The rare coexistence of the *cfiA* and *bft* gene among *B. fragilis* strains **(Paper II)**

During our study the same *bft* gene prevalence (14.0%) was found among the 486 *B. fragilis* isolates with a dominance of the *bft*1 than in earlier studies [Claros *et al*., 2000; Claros *et al*., 2006] however, we could not confirm the higher percentage of the carriage rate among blood culture isolates compared to isolates from other sources. The main finding of this part of our study was the confirmation of the co-existence of the *cfiA* and the *bft*1 genes in four isolates obtained from geographically different countries. We had data only for three of the four isolates about the source of isolation and those were collected from serious infections and despite of the fact that they did not have high MICs for imipenem (0.5-8 μg/ml) they harbored the "silent" *cfiA* gene beside one of the main pathogenicity gene known for the *B. fragilis* strains. During the comparison of the prevalence of the *bft* genes among the *cfiA*-negative (14,4%) and the *cfiA*-positive (9.3%) strains by using the Fischer's exact test did not reveal statistically significant differences, which means a basically equal distribution of the *bft* gene among the Division I and Division II isolates of *B. fragilis*. Very probably the *bft* gene-carrying conjugative transposon [Terhes *et al*., 2007] can easily enter Division II (*cfiA*-positive) *B. fragilis*

strains. The mobile property of the *bft* conjugative transposon is consistent with the fact that no clonality was observed among the *bft*-positive strains in earlier studies [Gutacker *et al*., 2000]. A recent report also presented the transferable nature of the *bft* genes since they were also detected among non-fragilis *Bacteroides* species from the microbiota of patients with colorectal carcinoma [Goodwin *et al*., 2012].

5.2.3. The prevalence of other antibiotic resistance genes among a subset of the 640 *B. fragilis* group strains (161) and the correlation of the presence of the genes with specific phenotypic resistance to different antibiotics **(Paper III, IV)**

For these 161 *B. fragilis* group strains we collected data for the presence of 16 different known resistance genes described earlier and tried to compare the phenotypic resistance pattern with the presence of the responsible genes. The same data were collected for further 53 recent *Bacteroides* isolates from Romania. No significant differences were found between the antibiotic resistance gene content of the earlier isolated consortia of strains from all over Europe and those isolated later in Romania.

In the case of certain genes (*cepA*, *cfxA*), our data confirmed earlier findings: *cepA* is very frequent among *Bacteroides* isolates and can be found among non-fragilis *Bacteroides* strains too [Mastrantonio *et al*., 1996; Boente *et al*., 2010; Lorenzo *et al*., 2012]. The prevalence of *cfxA* in this study (18% of all isolates harbored this gene) was similar to that published earlier being around 15-20% and it was shown not to be the only factor for cefoxitin resistance [Sóki *et al*., 2000; Avelar *et al*. 2003; García *et al*., 2008]. Among the strains with high cefoxitin MIC values (>64 μg/ml) the *cfxA* gene was often absent, a finding similar to that observed in an earlier study from our institute [Sóki *et al*., 2011].

Our study also clarified the contribution of other genes (*ermF* and *tetQ*) being the most frequently found resistance genes (26.5% and 81.9%, respectively) among all *Bacteroides* strains tested. The *ermF* gene was present in 76% of all clindamycin resistant *Bacteroides*, and in 100% of the three tigecycline resistant *B. fragilis* strains showing a close correlation with these resistance phenotypes. We also tested some recently described or unique resistance genes (*tetX*, *tetX1*, *tet36*, *tetM*, *ermB*, *ermG*, *linA, mefA* and *msrSA*) with varying correlation with phenotypic resistance to clindamycin or tigecycline of *B. fragilis* or non-fragilis *Bacteroides* species. However, we detected the co-localization of some genes (*ermG*, *mefA* and *msrSA*) in a proportion of clindamycin resistant strains (most probably due to harboring CTnGERM1) [Wang *et al*., 2003]. We

were not able to establish whether the genes in this combination were responsible for clindamycin resistance, but the results emphasize the prevalence of CTnGERM1 among our collection of strains [Wang *et al*., 2003]. Our data and those published earlier by Boente *et al.* (2010) about a smaller cohort of *Bacteroides* and *Parabacteroides* isolates might be useful in estimating the likelihood of emergence of the more resistant or multidrug-resistant *Bacteroides* strains in clinical settings. Multidrug-resistant *B. fragilis* and non-fragilis *Bacteroides* strains isolated increasingly in Europe [Wareham *et al*., 2005; Hartmeyer *et al*., 2012; Urbán *et al*., 2015] or other parts of the world [Rotimi *et al*, 1999; Sherwood *et al*., 2011; Kalapila *et al*., 2013] may pose a serious medical threat. In addition, since *Bacteroides* are important members of the normal intestinal microbiota, they are exposed to antibiotics used for various reasons by humans. They have also been observed as significant reservoirs and sources of antibiotic resistance genes. In our study, the *ermB* and *ermG* genes, which are characteristic for Gram-positive species [Nakajima, 1999], and the *tetX* and variant *tetX1* genes [Shoemaker *et al*, 2001], which code for aerobic type tetracycline oxidizing enzymes were found among both *B. fragilis* and non-fragilis *Bacteroides* isolates showing the possibility to transfer these genes in the microbiota. Some of the resistance genes examined by us were, however, found in susceptible strains as well, which raise the question of their genetic regulation. It is well known that *cepA*, *cfxA*, *cfiA*, *nim* and the *ermF* genes are activated by insertion sequence elements in *Bacteroides* strains, which may be the case in the other genes as well. Further more detailed studies are needed in the future to understand better the functionality of these genes.

The study reported here has provided a fairly complete picture concerning the connection of antibiotic resistance levels and the antibiotic resistance genes responsible for their development in Europe. However, it could also be a starting point for further investigations of the antibiotic resistance mechanisms of the *Bacteroides* species or for issues involving the prevalence of the antibiotic resistance genes isolated from the human intestinal microbiota.

6. CONCLUSIONS

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

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

6.3. Furthermore, the study reported here has provided data concerning the comparison of the antibiotic resistance levels and the presence of different antibiotic resistance genes responsible for their development in Europe using the largest collection of clinical isolates of this genus (214 isolates from 10 European countries, including Romania).
In the case of certain genes (*cepA, cfxA, cfiA*), our data confirmed earlier findings: (a)

cepA is very frequent among *Bacteroides* and can be found among non-fragilis *Bacteroides* too, (b) the prevalence of *cfxA* is around 15-20% (in our study 16%) and it is not the only factor for cefoxitin resistance, and (c) *cfiA* has a frequency around 5-7% (in our study 7%) in *B. fragilis* strains. The study also clarified the dominance of some genes (*ermF* and *tetQ*) behind the clindamycin and tigecyclin resistance, respectively. Some additional, known resistance genes such as *tetX*, *tetX1*, *tet36*, *tetM*, *ermB*, *ermG*, *msrSA*, *mefA* and *linA,* could also be detected both among *B. fragilis* and non-fragilis *Bacteroides* clinical isolates.

7. ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my advisor Professor Elisabeth Nagy for the continuous support of my PhD study and related research, for her continous patience, motivation, trust and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis.

I express my grateful thanks to Dr. József Sóki, for his essential professional advices, practical instructions and selfless support over the past years.

My sincere thanks also goes to Professor Judit Deák and Dr. Edit Urbán who provided me an opportunity to join the Institute of Clinical Microbilogy as a PhD student, and who gave access to the laboratory and research facilities.

I am grateful to Dr. Gabriella Terhes, Dr. Ulrik Stenz Justesen, Dr. Noémi Bartha, Dr. László Berecki, Viktor Sándor Fenyvesi, Anikó Keszőcze for their helpful cooperation and support.

I am grateful to all the members of the Institute of Clinical Microbiology to making possible my continuous research. I am especially grateful to Anita Bönde, Tünde Deák and Szilvia Batki for their excellent technical assistance, cooperation and support.

I am grateful to Dr. Nadja Shoemaker, Dr. Patrizia Spigaglia and Dr. Hiroshi Nikaido for sending control materials.

A special thanks to my family and friends for their love and support. Words cannot express how grateful I am to my father, my mother, my sister and my grandmothers for all of the sacrifices that you've made on my behalf.

I grateful to my beloved fiancé, György Móra. Thank you for supporting me for everything, and especially I can't thank you enough for encouraging me throughout this experience.

This work was supported by a grant from ESGAI (part of ESCMID), the Excellence Centre at the University of Szeged (TÁMOP-421B) and the Ministry of Education.

8. REFERENCES

Aldridge KE, Henderberg A, Schiro DD, *et al*. Susceptibility of *Bacteroides fragilis* group isolates to broad-spectrum β-lactams, clindamycin, and metronidazole: rates of resistance, cross-resistance, and importance of β-lactamase production. Adv Ther, 5:273-282, 1988.

Avelar KES, Otsuki K, Vicente ACP, *et al*. Presence of the *cfxA* gene in *Bacteroides distasonis*. Res Microbiol, 154:369-74, 2003.

Barry AL. Anaerobic susceptibility tests: differences between disc diffusion, agar dilution and broth microdilution test results. J Antimicrob Chemother, 25:875-7, 1990.

Bartha NA, Sóki J, Urbán E, *et al*. Investigation of the prevalence of *tetQ*, *tetX* and *tetX1* genes in *Bacteroides* strains with elevated tigecycline minimum inhibitory concentrations. Int J Antimicrob Agents, 38:522-5, 2011.

Bauer AW, Kirby WM, Sherris JC, *et al*. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 45:493-6, 1966.

Behra-Miellet J, Calvet L, Mory F, *et al*. Antibiotic resistance among anaerobic Gram-negative bacilli: lessons from a French multicentric survey. Anaerobe, 9:105-11, 2003.

Betriu C, Culebras E, Gómez M, *et al*. Resistance trends of the *Bacteroides fragilis* group over a 10-year period, 1997 to 2006, in Madrid, Spain. Antimicrob Agents Chemother, 52:2686–2690, 2008.

Boente RF, Ferreira LQ, Falcão LS, *et al*. Detection of resistance genes and susceptibility patterns in *Bacteroides* and *Parabacteroides* strains. Anaerobe, 16:190-4, 2010.

Brisson-Noël A and Courvalin P. Nucleotide sequence of gene *linA* encoding resistance to lincosamides in *Staphylococcus haemolyticus*. Gene, 43:247-53, 1986.

Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother, 39:1211-33, 1995.

Callihan DR and Nolte FS. Disc diffusion method to screen for high-level resistance to clindamycin and erythromycin in the *Bacteroides fragilis* group. Diagn Microbiol Infect Dis, 3:131-7, 1985.

Carlier JP, Sellier N, Rager MN, *et al*. Metabolism of a 5-nitroimidazole in susceptible and resistant isogenic strains of *Bacteroides fragilis*. Antimicrob Agents Chemother, 41:1495-1499, 1997.

Castellani A and Chalmers AJ. Manual of Tropical Medicine, 3rd ed. William Wood and Co., New York, 1919.

Citron DM, Ostovari MI, Karlsson A, *et al*. Evaluation of the E test for susceptibility testing of anaerobic bacteria. J Clin Microbiol, 29:2197–2203, 1991.

Claros MC, Claros ZC, Tang YJ, *et al*. Occurrence of *Bacteroides fragilis* enterotoxin gene-carrying strains in Germany and the United States. J Clin Microbiol, 38:1996-7, 2000.

Claros MC, Claros ZC, Hecht DW, *et al*. Characterization of the *Bacteroides fragilis* pathogenicity island in human blood culture isolates. Anaerobe, 12:17-22, 2006.

CLSI Clinical and Laboratory Standards Institute. Methods for antimicrobial susceptibility testing of anaerobic bacteria, 7th ed. Approved Standard, Document M11-A7, Wayne, PA: CLSI, 2007.

Cockerill FR 3rd. Genetic methods for assessing antimicrobial resistance, Antimicrob Agents Chemother, 43:199–212, 1999.

Deguchi T, Yasuda M, Nakano M, *et al*. Quinolone-resistant *Neisseria gonorrhoeae*: correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles. Antimicrob Agents Chemother, 40:1020–1023, 1996.

Deguchi T, Fukuoka A, Yasuda M, *et al*. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in quinolone-resistant clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother, 41:699–701, 1997.

Delahooke DM, Barclay GR, Poxton IR, *et al*. A re-appraisal of the biological activity of bacteroides LPS. J Med Microbiol, 42:102– 112, 1995.

Diniz CG, Farias LM, Carvalho MAR, *et al*. Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. J Antimicrob Chemother, 54:100–8, 2004.

Dix K, Watanabe SM, McArdle S, *et al*. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. J Clin Microbiol, 28:319–323, 1990.

Edwards R and Greenwood D. An investigation of β-lactamases from clinical isolates of *Bacteroides* species. J Med Microbiol, 36:89-95, 1992.

Edwards R. Resistance to beta-lactam antibiotics in *Bacteroides* spp. J Med Microbiol, 46:979-86, 1997.

Edwards R, Hawkyard CV, Garvey MT, *et al*. Prevalence and degree of expression of the carbapenemase gene (*cfiA*) among clinical isolates of *Bacteroides fragilis* in Nottingham, UK. J Antimicrob Chemother, 43:273-6, 1999.

Erikstrup LT, Danielsen TK, Hall V, *et al*. Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates. Clin Microbiol Infect, 18:E266-72, 2012.

Fernández-Canigia L, Litterio M, Legaria MC, *et al*. First national survey of antibiotic susceptibility of the *Bacteroides fragilis* group: emerging resistance to carbapenems in Argentina. Antimicrob Agents Chemother, 56:1309-14, 2012.

Finegold SM, Baron EJ, Wexler HM. A clinical Guide to Anaerobic Infections. Star Publishing Co., 1992.

Finegold SM. Anaerobic infections in humans: an overview. Anaerobe, 1:3-9,1995.

Franco AA, Cheng RK, Chung GT, *et al*. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. J Bacteriol, 181:6623-6633, 1999.

Franco AA. The *Bacteroides fragilis* pathogenicity island is contained in a putative novel conjugative transposon. J Bacteriol, 186:6077-6092, 2004.

Gal M and Brazier JS. Metronidazole resistance in *Bacteroides* spp. carrying *nim* genes and the selection of slow-growing metronidazole-resistant mutants. J Antimicrob Chemother, 54:109-16, 2004.

García N, Gutiérrez G, Lorenzo M, *et al*. Genetic determinants for *cfxA* expression in *Bacteroides* strains isolated from human infections. J Antimicrob Chemother, 62:942-7, 2008.

Goldstein EJ. Anaerobic bacteremia. Clin Infect Dis, 23:97–101, 1996.

Goodwin A, Badani R, Hechenbleikner E, *et al*. Isolation of novel, non-*Bacteroides fragilis* strains containing the *Bacteroides fragilis* toxin (Bft) locus from colorectal cancer patients. The 11th biennial congress of the anaerobe society of the Americas, 2012.

Gregory EM, Kowalski JB, Holdeman LV. Production and some properties of catalase and superoxide dismutase from the anaerobe *Bacteroides distasonis*. J Bacteriol, 129:1298–1302, 1977.

Gregory EM. Characterization of the O_2-induced manganese-containing superoxide dismutase from *Bacteroides fragilis*. Arch Biochem Biophys, 238:83–89, 1985.

Gutacker M, Valsangiacomo C, Piffaretti JC. Identification of two genetic groups in *Bacteroides* fragilis by multilocus enzyme electrophoresis: distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes. Microbiology, 146:1241-54, 2000.

Haggoud A, Reysset G, Azeddoug H, *et al*. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from *Bacteroides* strains and of a new insertion sequence upstream of the two genes. Antimicrob Agents Chemother, 38:1047-51, 1994.

Hartmeyer GN, Sóki J, Nagy E, *et al*. Multidrug-resistant *Bacteroides fragilis* group on the rise in Europe? J Med Microbiol, 61:1784-1788, 2012.

Horn R, Bourgault AM, Lamothel F. Disk diffusion susceptibility testing of the *Bacteroides fragilis* group. Antimicrob Agents Chemother, 31:1596–1599, 1987.

Ingham HR, Eaton S, Venables CW, *et al*. *Bacteroides fragilis* resistant to metronidazole after long-term therapy. Lancet, 1:214, 1978.

Jamal WY, Rotimi VO, Brazier JS, *et al*. Molecular characterization of nitroimidazole resistance in metronidazole-resistant *Bacteroides* species isolated from hospital patients in Kuwait. Med Princ Pract, 13:147-52, 2004.

Jenkins SG and Schuetz AN. Current Concepts in Laboratory Testing to Guide Antimicrobial Therapy, Mayo Clin Proc, 87:290–308, 2012.

Jouseimies-Somer H, Summanen P, Citron D, *et al*. Wadsworth-Ktl Anaerobic Bacteriology Manual 6th Edition. 2002.

Justesen US, Danielsen TK, Matuschek E, *et al*. The Brucella blood agar for disk diffusion antimicrobial susceptibility testing of the *Bacteroides fragilis* group? Clin Microbiol Infect, 17:S748, 2011.a

Justesen US, Matuschek E, Kahlmeter G. The EUCAST Mueller-Hinton fastidious agar for antimicrobial susceptibility testing of anaerobic bacteria? Clin Microbiol Infect, 17:S174, 2011.b

Kalapila A, Pergam S, Pottinger P, *et al*. Multidrug-resistant *Bacteroides fragilis*. Morb Mortal Wkly Rep, 62:694-696, 2013.

Kasper DL, Onderdonk AB, Crabb J, *et al*. Protective efficacy of immunization with capsular antigen against experimental infection with *Bacteroides fragilis*. J Infect Dis, 140:724-31, 1979.

Katsandri A, Papaparaskevas J, Pantazatou A, *et al*. Two cases of infections due to multidrug-resistant *Bacteroides fragilis* group strains. J Clin Microbiol, 44:3465-7, 2006.

Kwok Y-Y, Tally FP, Sutter VL, *et al*. Disk susceptibility testing of slow-growing anaerobic bacteria. Antimicrob Agents Chemother, 7:1–7, 1975.

Lee Y, Park Y, Kim MS, *et al*. Antimicrobial susceptibility patterns for recent clinical isolates of anaerobic bacteria in South Korea. Antimicrob Agents Chemother, 54:3993-7, 2010.

Ley RE, Backhed F, Turnbaugh P, *et al*. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA, 102:11070-11075, 2005.

Ley RE, Turnbaugh PJ, Klein S, *et al*. Microbial ecology: human gut microbes associated with obesity. Nature, 444:1022-1023, 2006.

Lorenzo M, García N, Ayala JA, *et al*. Antimicrobial resistance determinants among anaerobic bacteria isolated from footrot. Vet Microbiol, 157:112-8, 2012.

Löfmark S, Fang H, Hedberg M, *et al*. Inducible metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. Antimicrob Agents Chemother, 49:1253–6, 2005.

Löfmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. Clin Infect Dis, 50:S16-23, 2010.

Luu H, Thomsen HD, Hansen F, *et al*. Disc diffusion antimicrobial-susceptibility testing of the *Bacteroides fragilis* group using EUCAST clinical minimum inhibitory concentration (MIC) breakpoints. P1577, ECCMID 2013 Berlin, Germany, 2013.

Mastrantonio P, Cardines R, Spigaglia P. Oligonucleotide probes for detection of cephalosporinases among *Bacteroides* strains. Antimicrob Agents Chemother, 40:1014-6, 1996.

Miyamae S, Ueda O, Yoshimura F, *et al*. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides thetaiotaomicron*. Antimicrob Agents Chemother, 45:3341-6, 2001.

Moncrief JS, Obiso R, Jr, Barroso LA, *et al*. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. Infect Immun, 63:175-181, 1995.

Myers LL, Firehammer BD, Shoop DS, *et al*. *Bacteroides fragilis*: a possible cause of acute diarrheal disease in newborn lambs. Infect Immun, 44:241–244, 1984.

Myers LL, Shoop DS, Firehammer BD, *et al*. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease. J Infect Dis, 152:1344–1347, 1985.

Myers LL, Shoop DS, Stackhouse LL, *et al*. Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. J Clin Microbiol, 25:2330–2333, 1987.

Nagy E, Sóki J, Urbán E, *et al*. Occurrence of metronidazole and imipenem resistance among *Bacteroides fragilis* group clinical isolates in Hungary. Acta Biol Hung, 52:271-280, 2001.

Nagy E, Maier T, Urban E, *et al*. ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria. Species identification of clinical isolates of *Bacteroides* by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. Clin Microbiol Infect, 15:796-802, 2009.

Nagy E, Urbán E, Nord CE on behalf of the ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. Clin Microbiol Infect, 17:371–379, 2011.

Nakajima Y. Mechanisms of bacterial resistance to macrolid antibiotics. J Infect Chemother, 5:61-74, 1999.

Narikawa S, Suzuki T, Yamamoto M, *et al*. Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. J Antimicrob Chemother, 28:47–53, 1991.

Oitmaa M and Benn R. Sensitivity of *Bacteroides fragilis* to seven antimicrobial agents. Pathology, 13:697-703, 1981.

Olsson-Liljequist B and Nord CE. Methods for susceptibility testing of anaerobic bacteria, Clin Infect Dis, 18:S293-S296, 1994.

Onderdonk AB, Kasper DL, Cisneros RL, *et al*. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J Infect Dis, 136:82-9, 1977.

Pantosti A, Tzianabos AO, Reinap BG, *et al*. *Bacteroides fragilis* strains express multiple capsular polysaccharides. J Clin Microbiol, 31:1850-5, 1993.

Patel EH, Paul LV, Casanueva AI, *et al*. Overexpression of rhamnose catabolism regulatory protein. RhaR: a novel mechanism for metronidazole resistance in *Bacteroides thetaiotaomicron*. J Antimicrob Chemother, 69:267–73, 2009.

Patrick S. Variation of *Bacteroides fragilis* surface structures. Anaerobic pathogens, pp. 19–30, 1997.

Philips I, King A, Nord CE, *et al*. Antibiotic sensitivity of *Bacteroides fragilis* group in Europe. Eur J Clin Microbiol Infect Dis, 11:292-304, 1992.

Podglajen I, Breuil J, Bordon F, *et al*. A silent carbapenemase gene in strains of *Bacteroides fragilis* can be expressed after a one-step mutation. FEMS Microbiol Lett, 91:21–29, 1992.

Podglajen I, Breiul J, Collatz E. Insertion of a novel DNA sequence, IS*1186*, upstream of the silent carbapenemase gene *cfiA*, promotes expression of carbapenem resistance in clinical isolates of *Bacteroides fragilis*. Mol Microbiol, 12:105–114, 1994.

Podglajen I, Breuil J, Casin I, *et al*. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR-generated fragment patterns and insertion sequence content. J Bacteriol, 177:5270–5275, 1995.

Pumbwe L, Skilbeck CA, Nakano V, *et al*. Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis*. Microb Pathog, 43:78-87, 2007.

Rasmussen BA, Bush K, Tally FP. Antimicrobial resistance in *Bacteroides*. Clin Infect Dis, 16:S390-400, 1993.

Rasmussen BA, Bush K, Tally FP. Antimicrobial resistance in anaerobes. Clin Infect Dis, 24:S110-20, 1997.

Reid G. When microbe meets human. Clin Infect Dis, 39:827-830, 2004.

Reysset G, Haggoud A, Sebald M. Genetics of resistance of *Bacteroides* species to 5-nitroimidazole. Clin Infect Dis, 16:S401-3, 1993.

Reysset G. Genetics of 5-nitroimidazole resistance in *Bacteroides* species. Anaerobe, 2:59-69, 1996.

Roberts MC. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol Rev, 19:1-24, 1996.

Rotimi VO, Khoursheed M, Brazier JS, *et al*. Bacteroides species highly resistant to metronidazole: an emerging clinical problem? Clin Microbiol Infect, 5:166–169, 1999.

Rotimi VO, Verghese TL, Al-Sweih N, *et al*. Influence of five antianaerobic antibiotics on endotoxin liberation by gram-negative anaerobes. J Chemother, 12:40-47, 2000.

Ruimy R, Podglajen I, Breuil J, *et al*. A recent fixation of *cfiA* genes in a monophyletic cluster of *Bacteroides fragilis* is correlated with the presence of multiple insertion elements. J Bacteriol, 178:1914–1918, 1996.

Salyers AA, Shoemaker NB, Stevens AM, *et al*. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol Rev, 59:579–590, 1995.

Schapiro JM, Gupta R, Stefansson E, *et al*. Isolation of metronidazole-resistant *Bacteroides fragilis* carrying the *nimA* nitroreductase gene from a patient in Washington State. J Clin Microbiol, 42:4127–9, 2004.

Sherwood JE, Fraser S, Citron DM, *et al*. Multi-drug resistant *Bacteroides fragilis* recovered from blood and severe leg wounds caused by an improvised explosive device (IED) in Afghanistan. Anaerobe, 17:152-5, 2011.

Shoemaker NB, Vlamakis H, Hayes K, *et al*. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl Environ Microbiol, 67:561-8, 2001.

Simon GL and Gorbach SL. Intestinal flora in health and disease. Gastroenterology, 86:174-193, 1984.

Smalley D, Rocha ER, Smith CJ. Aerobic-type ribonucleotide reductase in the anaerobe

Bacteroides fragilis. J Bacteriol, 184:895-903, 2002.

Smith CJ. Nucleotide sequence analysis of Tn*4551* use of *ermFS* operon fusions to detect promoter activity in *Bacteroides fragilis*. J Bacteriol, 169:4589-4596, 1987.

Smith CJ and Parker AC. Identification of a circular intermediate in the transfer and transposition of Tn*4555*, a mobilizable transposon from *Bacteroides* spp. J Bacteriol, 175:2682–2691, 1993.

Smith CJ, Tribble GD, Bayley DP. Genetic elements of *Bacteroides* species: a moving story. Plasmid, 40:12-29, 1998.

Snydman DR, Jacobus NV, McDermott LA, *et al*. Lessons learned from the anaerobe survey: historical perspective and review of the most recent data (2005-2007). Clin Infect Dis, 50:S26-33, 2010.

Socransky SS, Smith C, Martin L, *et al*. "Checkerboard" DNA-DNA hybridization. Biotechniques, 17:788-92, 1994.

Sóki J, Urban E, Szöke I, *et al*. Prevalence of the carbapenemase gene (*cfiA*) among clinical and normal flora isolates of *Bacteroides* species in Hungary. J Med Microbiol, 49:427-30, 2000.

Sóki J, Fodor E, Hecht DW, *et al*. Molecular characterization of imipenem-resistant, *cfiA*-positive *Bacteroides fragilis* isolates from the USA, Hungary and Kuwait. J Med Microbiol, 53:413-419, 2004.a

Sóki J, Edwards R, Urbán E, *et al*. Screening of isolates from faeces for carbapenem-resistant *Bacteroides* strains; existence of strains with novel types of resistance mechanisms. Int J Antimicrob Agents. 24:450-4, 2004.b

Sóki J, Gal M, Brazier JS, *et al*. Molecular investigation of genetic elements contributing to metronidazole resistance in *Bacteroides* strains. J Antimicrob Chemother, 57:212-20, 2006.

Sóki J, Gonzalez SM, Urbán E, *et al*. Molecular analysis of the effector mechanisms of cefoxitin resistance among *Bacteroides* strains. J Antimicrob Chemother, 66:2492-500, 2011.

Steffens LS, Nicholson S, Paul LV, *et al*. *Bacteroides fragilis* RecA protein overexpression causes resistance to metronidazole. Res Microbiol, 161:346-54, 2010.

Stubbs SL, Brazier JS, Talbot PR. *et al*. PCR-restriction fragment length polymorphism analysis for identification of *Bacteroides* spp. and characterization of nitroimidazole resistance genes. J Clin Microbiol, 38:3209-3213, 2000.

Sutter VL, Kwok YY, Finegold SM. Standardized antimicrobial disc susceptibility testing of anaerobic bacteria. I. Susceptibility of *Bacteroides fragilis* to tetracycline. Appl Microbiol, 23:268-275, 1972.

Sutter VL, Kwok YY, Finegold SM. Susceptibility of *Bacteroides fragilis* to six antibiotics determined by standardized antimicrobial disc susceptibility testing. Antimicrob Agents Chemother, 3:188-193, 1973.

Sutter VL and Finegold SM. Susceptibility of anaerobic bacteria to 23 antimicrobial agents. Antimicrob Agents Chemother, 10:736–752, 1976.

Szöke I, Dósa E, Nagy E. Enterotoxigenic *Bacteroides fragilis* strains in Hungary. Anaerobe, 3: 87-89, 1997.

Tally FP, Stewart PR, Sutter VL, *et al*. Oxygen tolerance of fresh clinical anaerobic bacteria. J Clin Microbiol, 1:161–164, 1975.

Terhes G, Brazier JS, Sóki J, *et al*. Coincidence of *bft* and *cfiA* genes in a multi-resistant clinical isolate of *Bacteroides fragilis*. J Med Microbiol, 56:1416-1418, 2007.

Toprak NU, Uzunkaya OD, Soki J, *et al*. Susceptibility profiles and resistance genes for carbapenems (*cfiA*) and metronidazole (*nim*) among *Bacteroides* species in a Turkish University Hospital. Anaerobe, 18:169-71, 2012.

Trinh S and Reysset G. Detection by PCR of the *nim* genes encoding 5-nitroimidazole resistance in *Bacteroides* spp. J Clin Microbiol, 34:2078-2084, 1996.

Turner P, Edwards R, Weston V, *et al*. Simultaneous resistance to metronidazole, co-amoxiclav, and imipenem in clinical isolate of *Bacteroides fragilis*. Lancet, 345:1275-7, 1995.

Urbán E, Horváth Z, Sóki J, *et al*. First Hungarian case of an infection caused by multidrug-resistant *Bacteroides fragilis* strain. Anaerobe, 31:55-8, 2015.

Veillon MH and Zuber H. Recherches sur quelques microbes strictement anaerobes et leur role en pathologie. Arch Exp Med Path Anat, 10:517-545, 1898.

Veloo ACM, Welling GW, Degener JE. Antimicrobial susceptibility of clinically relevant

Gram-positive anaerobic cocci collected over a three-year period in the Netherlands. Antimicrob Agents Chemother, 55:1199–203, 2011.

Wang Y, Wang GR, Shelby A, *et al*. A newly discovered *Bacteroides* conjugative transposon, CTnGERM1, contains genes also found in gram-positive bacteria. Appl Environ Microbiol. 69:4595-603, 2003.

Wareham DW, Wilks M, Ahmed D, *et al*. Anaerobic sepsis due to multidrug-resistant *Bacteroides fragilis*: microbiological cure and clinical response with linezolid therapy. Clin Infect Dis, 40:e67-8, 2005.

Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. Clin Microbiol Rev, 20:593-621, 2007.

Whittle G, Hund BD, Shoemaker NB, *et al*. Characterization of the 13-kilobase *ermF* region of the *Bacteroides* conjugative transposon CtnDOT, Appl Environ Microbiol, 67:3488–3495, 2001.

Whittle G, Whitehead TR, Hamburger N, *et al*. Identification of a new ribosomal protection type of tetracycline resistance gene, *tet(36)*, from swine manure pits. Appl Environ Microbiol, 69:4151-8, 2003.

Wilkins TD, Holdeman LV, Abramson IJ, *et al*. Standardized single-disc method for antibiotic susceptibility testing of anaerobic bacteria, Antimicrob Agents Chemother, 1:451–459, 1972.

Wu S, Lim KC, Huang J, *et al*. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. Proc Natl Acad Sci USA, 95:14979-14984, 1998.

Wu S, Powell J, Mathioudakis N, *et al*. *Bacteroides fragilis* enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor-κB pathway. Infect Immun, 72:5832-5839, 2004.

Wybo I, Van den Bossche D, Soetens O, *et al*. Fourth Belgian multicentre survey of antibiotic susceptibility of anaerobic bacteria. J Antimicrob Chemother, 69:155-61, 2014.

Xu J and Gordon JI. Honor thy symbionts. Proc Natl Acad Sci USA, 100:10452-10459, 2003.

Yamazoe K, Kato N, Kato H, *et al*. Distribution of the *cfiA* gene among *Bacteroides fragilis* strains in Japan and relatedness of *cfiA* to imipenem resistance. Antimicrob

Agents Chemother, 43:2808-10, 1999.

PAPER I

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Molecular analysis of the carbapenem and metronidazole resistance mechanisms of *Bacteroides* strains reported in a Europe-wide antibiotic resistance survey

József Sóki*, Zsuzsa Eitel, Edit Urbán, Elisabeth Nagy, on behalf of the ESCMID Study Group on Anaerobic Infections[1]

Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged, Semmelweis 6, H-6725 Szeged, Hungary

ARTICLE INFO

Article history:
Received 8 September 2012
Accepted 3 October 2012

Keywords:
Antibiotic resistance mechanisms
Bacteroides fragilis
cfiA
nim
Insertion sequence (IS) elements

ABSTRACT

Here we examine the carbapenem and metronidazole resistance mechanisms of 640 *Bacteroides* strains reported in the 2008–2009 European antibiotic susceptibility survey. Of the 22 strains with elevated imipenem minimum inhibitory concentrations (≥4 μg/mL), 10 were *cfiA*-positive and out of these 5 carried activating insertion sequence (IS) elements in the upstream regions of the *cfiA* genes. However, resistant strains with *cfiA* genes but with no activating IS elements were found ($n = 2$) as well as a resistant strain with no *cfiA* gene. In the former the resistance phenotypes by Etest were heterogeneous, whilst in the latter no carbapenemase production was seen; both mechanisms have been rarely observed, examined and characterised. Interestingly, few ($n = 3$) *nim*-positive strains were found, including one metronidazole-resistant strain harbouring *nimE* activated by IS*Bf*6, and two susceptible strains harbouring chromosomally located *nim* genes.

1. Introduction

Bacteroides spp. represent one of the most significant groups of anaerobic bacteria. They are important constituents of the intestinal microbiota, from where they can cause severe anaerobic infections ranging from those of the soft tissue and upper respiratory tract to sepsis and various abscesses [1]. *Bacteroides* spp. can harbour the highest number of antibiotic resistance mechanisms and have the highest antibiotic resistance prevalences among all pathogenic anaerobes [2]. Because of their special and usually long culture requirements, temporary records of antibiotic resistance rates is considered a good and recommended practice worldwide. Such monitoring was performed mostly in the USA [3] and Europe [4], the latter under the organisation of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Antimicrobial Resistance in Anaerobic Bacteria (ESGARAB), whose name was changed to the ESCMID Study Group on Anaerobic Infections to cover a broader interest. The general trend is almost 100% resistance to penicillins, cephalosporins and tetracycline, a rising moderate resistance prevalence to cefoxitin, clindamycin and moxifloxacin, and very low prevalences for carbapenems, β-lactam/β-lactamase combinations, metronidazole and tigecycline [3,4]. Following antibiotic resistance monitoring for *Bacteroides* in 2000, molecular analyses were carried out to determine the metronidazole and carbapenem resistance mechanisms [5,6]. These investigations demonstrated the roles of the *nim* and *cfiA* genes and their activating insertion sequence (IS) elements in metronidazole and carbapenem resistance mechanisms, respectively.

Carbapenem-resistant *Bacteroides* isolates usually belong to the *Bacteroides fragilis* group, with the *cfiA* resistance gene being chromosomal and the majority of *cfiA*-positive strains being susceptible phenotypically because of the lack of upregulating IS elements [1]. The best-characterised metronidazole resistance mechanism among *Bacteroides* strains is due to the *nim* genes (*nimA–F*) that may occur in all *Bacteroides* species, and they are either located on well-characterised plasmids or on the chromosome. The majority of *nim*-positive *Bacteroides* isolates studied harbour a *nim* gene and a corresponding IS element pair [6]. It is of interest that the *cfiA*-positive *B. fragilis* isolates form a subgroup within this species. The *cfiA*-negative and *cfiA*-positive strains are therefore often classified as Division I and II, respectively, and can be distinguished by differences in DNA–DNA homology rates and by molecular typing methods such as randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), ribotyping, multilocus enzyme electrophoresis, sequence typing and

* Corresponding author. Tel.: +36 62 545 712; fax: +36 62 545 712.
E-mail address: soki.jozsef@med.u-szeged.hu (J. Sóki).

[1] ESCMID Study Group on Anaerobic Infections: Denis Piérard, Youri Glupczynski, Claire Nonhoff, Margareta Ieven (Belgium); Smilja Kalenić (Croatia); Eva Chmelařova (Czech Republic); Eija Können (Finland); Laurent Calvet, Luc Dubreuil (France); Erika Dósa (Germany); Joseph Papaparaskevas (Greece); Lenke Szikra, Cecilia Miszti, Gabriella Terhes (Hungary); Annarita Mazzariol (Italy); Jordi Vila (Spain); Maria Hedberg (Sweden); John Degener, Linda Wildeboer-Veloo (The Netherlands); and Nezhat Güler, Sabiha Güner, Nurver Ulger (Turkey).

0924-8579/$ – see front matter © 2012 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.
http://dx.doi.org/10.1016/j.ijantimicag.2012.10.001

Table 1
Primers and PCR conditions originally designed and applied in this study.

PCR	Primers	Sequence 5′→3′	PCR conditions
cfiA	cfiA-RT1 cfiA-RT2	AATCGAAGGATGGGGTATGG CGGTCGGTGAATCCGTGAAT	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 59 °C for 1 min, 72 °C for 30 s; melting 72–95 °C
nim[a]	nim3 nim5	ATGTTCAGAGAAATGCGGCGTAAGCG GCTTCCTTGCCTGTCATGTGCTC	95 °C for 10 min; 35 cycles of 95 °C for 30 s, 62 °C for 1 min, 72 °C for 1 min; melting 72–95 °C

[a] The method of Trinh and Reysset was adapted to real-time PCR [13].

matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry [7–12]. The Ambler class A cephalosporinase gene, *cepA*, and the enterotoxin *bft* genes were reported to occur exclusively in Division I strains [10].

This study investigated the prevalences of the *cfiA* and *nim* genes, the imipenem and metronidazole resistance mechanisms in the majority of *Bacteroides* strains reported in the 2008 European *Bacteroides* antibiotic resistance survey.

2. Materials and methods

2.1. Bacterial strains and cultivation

A total of 640 isolates belonging to the *Bacteroides* and *Parabacteroides* genera (486 *B. fragilis*, 54 *Bacteroides thetaiotaomicron*, 36 *Bacteroides ovatus*, 33 *Bacteroides vulgatus*, 8 *Bacteroides uniformis*, 7 *Parabacteroides distasonis*, 4 *Parabacteroides merdae*, 3 *Bacteroides eggerthii*, 3 *Bacteroides massiliensis*, 3 *Bacteroides nordii*, 2 *Bacteroides caccae* and 1 *Bacteroides stercoris*) were analysed from the collection sent to the central laboratory (Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary) for the 2008–2009 European *Bacteroides* antibiotic susceptibility survey (participating countries: Belgium, Croatia, Czech Republic, Finland, France, Germany, Greece, Hungary, Italy, Spain, Sweden, The Netherlands and Turkey). Isolate identification was carried out by routine clinical methods. Strains were stored at −70 °C in CryoBank vials (Mast Diagnostica, Rheinfeld, Germany) and were cultivated at 37 °C anaerobically on Columbia agar supplemented with 5% (v/v) sheep blood, 5 g/L haemin and 1 g/L vitamin K_1, or in BHIS broth [brain–heart infusion broth supplemented with 0.5% (w/v) yeast extract, 5 g/L haemin and 1 g/L vitamin K_1] in an anaerobic cabi net (Concept 400; Ruskinn Technology Ltd., Bridgend, UK) under gas composition of 85% N_2, 10% H_2 and 5% CO_2 for 48 h. Antibioti resistance results were obtained from the susceptibility measure ments done previously by the agar dilution method [4] or by Etes (bioMérieux, Marcy-l'Étoile, France) as recommended by the sup plier. The following control strains were used: *B. fragilis* TAL363 (*cfiA*); *B. fragilis* 638R (pIP417) (*nimA*); *B. fragilis* BF-8 (*nimB*); *B. frag ilis* 638R (pIP419) (*nimC*); *B. fragilis* 638R (pIP421) (*nimD*); and *B fragilis* 388 (*nimE*).

2.2. Real-time PCR detection of the cfiA and nim genes

Bacterial template DNA samples for the real-time PCR analy sis were prepared by incubating 100 μL of 0.5 McFarland turbidity suspensions at 100 °C for 10 min, which were stored at −30 °C unti use. Real-time PCR experiments were carried out in an MXPro3000 instrument (Stratagene, Santa Clara, CA) with the following reac tion setup: 1× MasterMix [iQ™ (Bio-Rad Hungary, Budapest Hungary) with 1× EvaGreen® (Biotium Inc., Hayward, CA) for *nim* or Brilliant III (Stratagene/Agilent, Santa Clara, CA) for *cfiA* and *bft* 0.7 μM of each primer and 2 μL of template DNA preparation i 10 μL final volumes in 96-well PCR reaction plates. The nucleotid sequences of the newly used primers and the cycling condition chosen during this study are shown in Table 1. Positive reaction were identified by the starting amplification cycle, melting curve showing the correct melting temperatures, and in rare cases when it was required to compare the size of the products with those o the positive controls in 1.2% agarose gel electrophoresis.

Table 2
Analysis of the imipenem resistance mechanism of strains with elevated imipenem minimum inhibitory concentrations (MICs) (≥4 μg/mL).

Strain	Imipenem MIC (μg/mL)	*cfiA*	Upstream region	Mechanism
Bacteroides fragilis SW42	4	–	–	Other[a]
B. fragilis SW46	4	–	–	Other
B. fragilis SW83	4	–	–	Other
B. fragilis TR38	4	–	–	Other
B. fragilis HU25	4	–	–	Other
B. fragilis FI63	4	–	–	Other
Bacteroides eggerthii GR67	4	–	–	Other
Bacteroides thetaiotaomicron BEM28	4	–	–	Other
Parabacteroides merdae GR70	4	–	–	Other
B. fragilis DE14	4	+	280 bp[b]	Silent with increased MIC
B. fragilis HU51	4	+	280 bp[b]	Silent with increased MIC
B. fragilis IT15	4	+	IS*4351*	IS-activated
Bacteroides stercoris HU59	8	–	–	Other
B. thetaiotaomicron BEA22	8	–	–	Other
B. fragilis HU92	8	+	280 bp[b]	Silent with increased MIC
B. fragilis TR27	16	+	IS*1187*	IS-activated
B. fragilis TR31	16	+	IS*1187*	IS-activated
B. fragilis HU61	32	+	280 bp[b]	Heteroresistant
B. fragilis NLH3	>32	+	IS*8TT*	IS-activated
B. fragilis FR41	>32	+	280 bp[b]	Heteroresistant
B. fragilis FI87	>32	+	IS*6148*	IS-activated
B. fragilis FI37	>32	–	–	Other

[a] The effects are not caused by *cfiA*.
[b] The 280-bp PCR fragment displays no insertion upstream of *cfiA*.

2.3. Analysis of the carbapenem and metronidazole resistance mechanisms by molecular methods

An analysis of the carbapenem and metronidazole resistance mechanisms was carried out as previously described [14,15]. Imipenemase activities were recorded in a 50 mM $NaPO_4$ (pH 7.0) buffer using sonicated cell extracts and 0.1 mM imipenem by following absorbance changes at 299 nm. Protein concentrations were measured with a Quant-iT™ Protein Assay Kit using a Qubit® Mini Fluorometer (Life Technologies Hungary Ltd., Budapest, Hungary). Tazobactam (10 μg/mL) or 10 mM ethylene diamine tetra-acetic acid (EDTA) were used to inhibit the enzymes, and imipenemase activity was expressed as 1 nmol hydrolysed imipenem/min (1 U) standardised by the protein concentration of the sonicates. Nucleotide sequencing was performed using an automated sequencer as described previously [15]. The novel nucleotide sequence of IS*Bf11* was deposited in the GenBank database under accession no. GQ449386.

3. Results and discussion

3.1. Resistance mechanisms of Bacteroides strains with elevated imipenem minimum inhibitory concentrations (MICs)

Of the 640 *Bacteroides* strains included in this study, 22 had imipenem MICs ≥ 4 μg/mL. Of the 486 *B. fragilis* strains examined, 43 were *cfiA*-positive, and from the 640 *Bacteroides* isolates examined 22 and 7 had imipenem MICs ≥4 μg/mL and ≥16 μg/mL, respectively. No non-*fragilis Bacteroides* strains were resistant to imipenem and only one *cfiA*-negative *B. fragilis* isolate was resistant. The results are summarised in Table 2. Of the 10 *B. fragilis* strains with elevated imipenem MICs (4–8 μg/mL), 4 (40.0%) were *cfiA*-positive, whilst 6 (85.7%) of the 7 imipenem-resistant (MIC ≥ 16 μg/mL) *B. fragilis* isolates were *cfiA*-positive. Among the strains with elevated MICs and with *cfiA* genes, one harboured an IS element upstream of *cfiA* (*B. fragilis* IT15), and among the *cfiA*-positive and imipenem-resistant strains four harboured IS elements upstream of the resistance gene (Table 2). The remaining two *cfiA*-positive isolates that were imipenem-resistant but without activating IS elements upstream of *cfiA* displayed a heterogeneous resistance phenotype using the imipenem Etest (see the example in Fig. 1).

This study yielded similar prevalence values for the molecular mechanisms of imipenem resistance of *B. fragilis* strains as those in previous studies. Among the highly imipenem-resistant strains (MIC ≥ 16 μg/mL), the *cfiA* genes are activated by IS elements (4 of 6 *cfiA*-positive), and among strains with elevated imipenem MICs (≥4 μg/mL) the *cfiA* genes were enriched (26.7% compared with the commonly found 2–8%). The types of *cfiA*-activating IS elements were IS*1187* ($n = 2$), IS*614B* ($n = 1$), and a novel IS element (n = IS*Bf11*; GenBank accession no. GQ449386) for *B. fragilis* H3 that had 77% homology compared with IS*614B* (Table 2). *Bacteroides fragilis* IT15 harboured IS*4351* upstream of the *cfiA* gene, but its imipenem MIC was low (4 μg/mL). This latter finding is in accordance with that of Podglajen et al. [8] who found that *B. fragilis* strains carrying IS*4351* upstream of the *cfiA* genes also tended to have low imipenem MICs (16 μg/mL) compared with other IS elements (IS*942* and IS*1186*; MICs ≥ 64 μg/mL). Previously we detected a probably low-activity *Bacteroides* promoter-like sequence in the upstream regions of the *cfiA* genes. According to this, our study and other studies detected elevated imipenemase activities in 'silent' *cfiA*-positive strains that could account for the imipenem MICs in such strains [14,16]. However, some highly imipenem-resistant strains ($n = 2$) were also genetically silent, their *cfiA* genes not being activated by IS elements (Table 2). In these cases Etest susceptibility tests detected

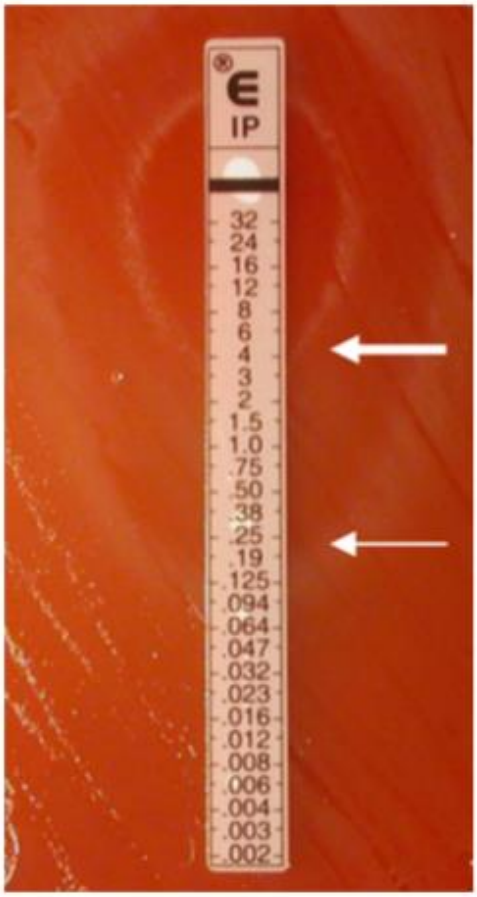


Fig. 1. Heterogeneous imipenem-resistant phenotype of *Bacteroides fragilis* FR41 detected by Etest. The first (0.25 μg/mL) and second (4 μg/mL) inhibition zones are marked by thin and thicker arrows, respectively.

heterogeneous resistance phenotypes (Fig. 1) where, inside of confluent inhibition zones, resistant colonies or growth appeared. This phenomenon can be explained by activation of the *cfiA* genes by an as yet unidentified mechanism that boosts the carbapenemase activity of the strains. We previously described such heteroresistant strains from human faeces whose imipenem MICs and imipenemase activities displayed a relation. In contrast, the *cfiA* genes were not activated by IS elements [16]. For *Bacteroides*, we detected heterogeneously cefoxitin-resistant strains and hypothesised that the copy number of the corresponding *cfxA* resistance gene might be important [17].

A *cfiA*-negative but imipenem-resistant *B. fragilis* isolate was identified in this study (*B. fragilis* FI37; Table 2). Such strains were also found previously, but the exact carbapenem resistance mechanism for these strains has not yet been clarified [18,19]. An imipenemase assay of this strain did not reveal any activity, whilst the control *B. fragilis* TAL3636 strain produced 41.0 U/mg imipenemase activity that was inhibited by EDTA. The probable resistance mechanisms are penicillin-binding protein (PBP) affinity or permeability changes.

A strain-dependent role for PBPs in the case of eight *B. fragilis* strains with various imipenem MICs (0.12–16 μg/mL) was reported

previously [19], and mutations of an endogenous efflux system (*bmeABC*) also affected the carbapenem susceptibilities of the carrying strains [20].

3.2. Detection of *nim* genes and their relation to metronidazole resistance

Of the 640 *Bacteroides* strains, 21 had reduced susceptibility to metronidazole (MIC ≥ 4 μg/mL) and only 3 (*B. fragilis* IT724 and IT797 and *B. thetaiotaomicron* HU66) harboured *nim* genes, with the following metronidazole MICs: 0.125 μg/mL (*B. fragilis* IT797), 1 μg/mL (*B. fragilis* IT724) and 256 μg/mL (*B. thetaiotaomicron* HU66). An examination of the *nim*-mediated resistance mechanisms revealed that *B. fragilis* IT797 and IT724 harboured chromosomal *nimA* and *nimC* genes, respectively. By contrast, the *nimE* gene of *B. thetaiotaomicron* HU66 was located on an 8.3 kb (pBF388c-like) [15] plasmid and was activated by IS*Bf6* (data not shown). No *nim*-specific plasmids were detected in the two other strains (a 5.6 kb class III plasmid and no plasmid content were characteristic for *B. fragilis* IT797 and IT724, respectively). Furthermore, *B. fragilis* IT797 harboured IS*1168* and IS*1170*, but these elements could not be mapped to the *nimA* gene by PCR mapping. From these results, it appears that the situation with *nim*-mediated metronidazole-resistant *Bacteroides* strains has changed in Europe compared with the previous study where 43 *Bacteroides* strains with reduced metronidazole susceptibility (MICs ≥ 4 μg/mL; 3.3%) and 30 (2.0%) *nim*-positive strains were found [6]. The current situation in Europe is reminiscent of that in the USA where *nim* genes and metronidazole resistances were scarce for a long time [21]. The *nim*-negative but metronidazole-resistant *Bacteroides* strains found in the current study may have other resistance mechanisms (reduced uptake, nitroreductase and pyruvate–ferredoxin oxidoreductase activities, increased lactate dehydrogenase activity, or mutations that alter the carbohydrate utilisation affecting the redox state) which shortcut the detrimental cellular effects of this drug [2,22–24].

In conclusion, these results confirmed the present view of carbapenem and metronidazole resistance mechanisms of *Bacteroides* spp. but also provide new information regarding their current state and epidemiology in Europe in addition to newly described mechanisms such as non-carbapenemase-mediated imipenem resistance and chromosomal *nim* genes.

Funding: This study was supported by the Excellence Centre of the University of Szeged (Szeged, Hungary) (TÁMOP 421B), the Hungarian National Research Fund (OTKA 69044) and the ESCMID Study Group of Anaerobic Infections.

Competing interests: None declared.

Ethical approval: Not required.

References

[1] Wexler HM. *Bacteroides*: the good, the bad and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.

[2] Rasmussen BA, Bush K, Tally FP. Antimicrobial resistance in *Bacteroides*. Clin Infect Dis 1993;16(Suppl. 4):S390–400.

[3] Snydman DR, Jacobus NV, McDermott LA, Golan Y, Hecht DW, Goldstein EJC, et al. Lessons learned from the anaerobe survey: historical perspective and review of most recent data (2005–2007). Clin Infect Dis 2010;50(Suppl 1):S26–33.

[4] Nagy E, Urbán E, Nord CE; ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. Clin Microbiol Infect 2011;17:371–5.

[5] Sóki J, Edwards R, Hedberg M, Fang H, Nagy E, Nord CE. Examination of *cfiA* mediated carbapenem resistance in *Bacteroides fragilis* strains from a European antibiotic susceptibility survey. Int J Antimicrob Agents 2006;28:497–502.

[6] Löfmark S, Fang H, Hedberg M, Edlund C. Inducible metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. Antimicrob Agents Chemother 2005;49:1253–6.

[7] Johnson JL. Taxonomy of the *Bacteroides*. I. Deoxyribonucleic acid homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. Int J Syst Bacteriol 1978;28:245–56.

[8] Podglajen I, Breuil J, Casin I, Collatz E. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR generated fragment patterns and insertion sequence content. J Bacteriol 1995;177:5270–5.

[9] Ruimy R, Podglajen I, Breuil J, Christen R, Collatz E. A recent fixation of *cfiA* gene in a monophyletic cluster of *Bacteroides fragilis* is correlated with the presence of multiple insertion elements. J Bacteriol 1996;178:1914–8.

[10] Gutacker M, Valsangiacomo C, Piffaretti JC. Identification of two genetic groups in *Bacteroides fragilis* by multilocus enzyme electrophoresis: distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes. Microbiology 2000;146:1241–54.

[11] Wybo I, De Bel A, Soetens O, Echahidi F, Vandoorslaer K, van Cauwenberge M, et al. Differentiation of *cfiA*-negative and *cfiA*-positive *Bacteroides fragilis* isolates by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 2011;49:1961–4.

[12] Nagy E, Becker S, Sóki J, Urbán E, Kostrzewa M. Differentiation of division I (*cfiA* negative) and division II (*cfiA*-positive) *Bacteroides fragilis* strains by matrix assisted laser desorption/ionization time of-flight mass spectrometry. J Med Microbiol 2011;60:1584–90.

[13] Trinh S, Reysset G. Detection by PCR of the *nim* genes encoding 5-nitroimidazole resistance in *Bacteroides* spp. J Clin Microbiol 1996;34:2078–84.

[14] Sóki J, Fodor E, Hecht DW, Edwards R, Rotimi VO, Kerekes I, et al. Molecular characterization of imipenem-resistant *cfiA*-positive *Bacteroides fragilis* isolates from the USA, Hungary and Kuwait. J Med Microbiol 2004;53:413–9.

[15] Sóki J, Gal M, Brazier JS, Rotimi VO, Urbán E, Nagy E, et al. Molecular investigation of genetic elements contributing to metronidazole resistance in *Bacteroides* strains. J Antimicrob Chemother 2006;57:212–20.

[16] Sóki J, Edwards R, Urbán E, Fodor E, Beer Z, Nagy E. Screening of isolates from faeces for carbapenem-resistant *Bacteroides* strains; existence of strains with novel types of resistance mechanisms. Int J Antimicrob Agents 2004;24:450–4.

[17] Sóki J, Gonzalez SM, Urbán E, Nagy E, Ayala JA. Molecular analysis of the effector mechanisms of cefoxitin resistance among *Bacteroides* strains. J Antimicrob Chemother 2011;66:2492–500.

[18] Yamazoe K, Kato N, Kato H, Tanaka K, Katagiri Y, Watanabe K. Distribution of the *cfiA* gene among *Bacteroides fragilis* strains in Japan and relatedness of *cfiA* to imipenem resistance. Antimicrob Agents Chemother 1999;43:2808–10.

[19] Ayala J, Quesada A, Vadillo S, Criado J, Píriz S. Penicillin-binding proteins of *Bacteroides fragilis* and their role in resistance to imipenem of clinical isolates. J Med Microbiol 2005;54:1055–64.

[20] Pumbwe L, Ueda O, Yoshimura F, Chang A, Smith RL, Wexler HM. *Bacteroides fragilis* BmeABC efflux systems additively confer intrinsic antimicrobial resistance. J Antimicrob Chemother 2006;58:37–46.

[21] Schapiro JM, Gupta R, Stefansson E, Fang FC, Limaye AP. Isolation of metronidazole-resistant *Bacteroides fragilis* carrying the *nimA* nitroreductase gene from a patient in Washington State. J Clin Microbiol 2004;42:4127–9.

[22] Narikawa S, Suzuki T, Yamamoto M, Nakamura M. Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. Antimicrob Chemother 1991;28:47–53.

[23] Diniz CG, Farias LM, Carvalho MAR, Rocha ER, Smith CJ. Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. J Antimicrob Chemother 2004;54:100–8.

[24] Patel EH, Paul LV, Casanueva AI, Patrick S, Abratt VR. Overexpression of rhamnose catabolism regulatory protein, RhaR: a novel mechanism for metronidazole resistance in *Bacteroides thetaiotaomicron*. J Antimicrob Chemother 2009;69:267–73.

PAPER II

Anaerobe 23 (2013) 70–73

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular biology, genetics and biotechnology

Occurrence and analysis of rare *cfiA–bft* doubly positive *Bacteroides fragilis* strains

József Sóki*, Zsuzsa Eitel, Gabriella Terhes, Elisabeth Nagy, Edit Urbán, on behalf of the ESCMID Study Group on Anaerobic Infections[1]

Institute of Clinical Microbiology, Albert Szent-Györgyi Clinical Center, Faculty of Medicine, University of Szeged, Semmelweis 6, H-6725 Szeged, Hungary

ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form
7 June 2013
Accepted 13 June 2013
Available online 22 June 2013

Keywords:
Bacteroides fragilis
bft1
cfiA
Conjugative transposons
Enterotoxin

ABSTRACT

We detected four *cfiA–bft1* doubly positive *Bacteroides fragilis* strains out of 486 *B. fragilis* isolates analyzed for antibiotic susceptibilities and antibiotic resistance genes from a recent pan-European survey. The prevalence of the enterotoxin *bft* genes was roughly equal among *cfiA*-negative and -positive *B. fragilis* strains. We also demonstrated that the *cfiA–bft* doubly positive strains had the most common *B. fragilis* genomic pattern (I.1.). Thus we concluded that the *bft*-carrying CTn*86* conjugative transposons are mobile accounting for this unexpected simultaneous occurrence of the *cfiA* and *bft* genes.

1. Introduction

The *Bacteroides* species are important constituents of the normal human intestinal microbiota, exerting beneficial effects on the host physiology. Besides the commensal and symbiotic interactions, they can be opportunistic pathogens in such anaerobic infections as intra-abdominal and soft tissue abscesses and rarely even sepsis. The most pathogenic species is *Bacteroides fragilis*, which accounts for about 5% of the total *Bacteroides* flora in the large bowel [1], but for about 60–80% of *Bacteroides* infections. Additionally, some *B. fragilis* strains have been found to cause intestinal pathogenicity, diarrhea, especially in young mammals (enterotoxinogenic *B. fragilis*, ETBF) [2]. The effector toxin in this case is a metallo-protease, fragilysin, which is able to give rise to cleavage of the E-cadherin protein of the zonula adhaerens epithelial barrier and reorganization of the actin cytoskeleton of the intestinal epithelium, thereby causing the symptoms [3]. The fragilysin genes, *bft1–3*, have been demonstrated to be located on a ca. 6 kb portion (BfPAI) of a conjugative transposon, CTn*86* [4–7].

For *B. fragilis* one of the most effective treatment options is the carbapenems, against which only 1% of the strains are resistant [8]. In these cases, production of a metallo-β-lactamase is the main resistance mechanism. This enzyme is produced from the chromosomal *cfiA* (*ccrA*) gene and, for high-level resistance to occur, insertion sequence (IS) elements up-regulate the gene via outward-oriented promoters; otherwise the IS-less *cfiA*-positive strains remain susceptible. Such strains account for the majority of the *cfiA*-positive cases [2,8]. It is of interest that the *cfiA*-positive *B. fragilis* isolates form a subgroup within this species. The *cfiA*-negative and the *cfiA*-positive strains are therefore often classified as Division I and II, respectively, and can be distinguished by differences in DNA–DNA homology rates and by molecular typing methods such as RAPD-PCR, ribotyping, multilocus enzyme electrophoresis, sequence typing and MALDI-TOF mass spectrometry [9–15]. The Ambler Class A cephalosporinase gene, *cepA*, and the enterotoxin *bft* genes have been reported to occur exclusively in Division I strains [12]. However, a *B. fragilis* isolate (*B. fragilis* WI1) which was found to harbor the *cfiA* and a *bft* gene simultaneously was thought to be an exception [16]. An alternative subgrouping of *B. fragilis* isolates based on the characteristics of the CTn*9343* and

* Corresponding author. Tel./fax: +36 62 545712.
E-mail addresses: soki.jozsef@med.u-szeged.hu, dr.soki.jozsef@invitel.hu (J. Sóki).

[1] Denis Piérard, Youri Glupczynski, Claire Nonhoff, Margareta Ieven (Belgium), Smilja Kalenić (Croatia), Eva Chmelařova (Czech Republic), Eija Könönen (Finland), Laurent Calvet, Luc Dubreuil (France), Erika Dósa (Germany), Joseph Papaparaskevas (Greece), Lenke Szikra, Cecilia Miszti (Hungary), Annarita Mazzariol (Italy), Jordi Vila (Spain), Maria Hedberg (Sweden), John Degener, Linda Wildeboer-Veloo (The Netherlands), Nezhat Güler, Sabiha Güner, Nurver Ulger (Turkey).

1075-9964/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.anaerobe.2013.06.008

CTn*86* conjugative transposons, two related possibly mobile genetic elements, have been established. Thus the *B. fragilis* strains can be placed into 7 groups (I.1–3., II. and III.1–3.) based on the presence or absence of the these CTns, of the *bft*-carrying 6 kb portion (BfPAI) and of another insertional element that is 7 kb [6].

In this study, we report on the detection, prevalence and analysis of *B. fragilis* strains that were *bft–cfiA* doubly positives from a subset of *B. fragilis* isolates collected for a European antibiotic resistance survey in 2008–2009 and tested for antibiotic resistance mechanisms. The previously identified *cfiA* and *bft1*-positive *B. fragilis* WI1 (R19811) was also included.

2. Materials and methods

486 *B. fragilis* isolates were examined in the study. The template DNA preparation and detection of the *cfiA* genes were carried out as previously described [17]. The enterotoxin *bft* genes were detected as follows. Primers were designed (bft-F: CGAACTCGGTTTATGCAGTT, and bft-R GGATACATCAGCTGGGTTGT) for RT-PCR (Primer3 software, http://frodo.wi.mit.edu/primer3/). PCR mixes contained 5 μl iQ mastermix (BioRad) 0.7 μM primers and 2 μl template DNA, and were subjected to cycling (95 °C 5 min 1×; 95 °C 15 s, 56 °C 1 min, 72 °C 30 s, 35×), in an RT-PCR instrument (Mx3000Pro, Stratagene), with subsequent melting-curve detection. The *bft* alleles of the positive strains for the RT-PCR were determined by PCR RFLP. Using the BTT1 (CATGTTCTAATGAAGCTGATTC) and BTT2 (ATCGCCATCTGCTGTTTCCC) primers the entire *bft* genes were amplified in end-point PCRs (95 °C 10 min 1×; 95 °C 30 s, 62 °C 1 min, 72 °C 1 min, 35×), the PCR products were purified with the HighPure PCR Cleanup Kit (Roche) after agarose electrophoresis and the pure products were digested with *Mbo*I restriction enzyme. The final products were analyzed by 1.5% agarose gel electrophoresis in TBE buffer, using 0.5 μg/ml ethidium bromide and UV visualization. The PCR products using the BTT1 and BTT2 primers were used in PCR RFLP and the expected sizes of the digested fragments were as follows: 839 and 310 bp (*bft1*), 575, 453 and 111 bp (*bft2*) 839, 189 and 111 bp (*bft3*), respectively. *B. fragilis* VPI 13783 (*bft1*) and *B. fragilis* GAI 96478 (*bft3*) were used as positive controls. The CTn*86* elements of the *cfiA–bft* doubly positive isolates were characterized as described by Buckwold et al. [6] to examine possible molecular differences between CTn*86* and CTn*43493* (the left end of CTn86, and the 7-kb insertion in CTn*4393*, Table 1) and differences among the CTn*86* elements in these strains (insertion or lack of the BfPAI, Table 1). The excised circular intermediates of the CTn*86* conjugative transposons were induced by half MIC moxifloxacin exposure according to Franco [5] and in one case (*B. fragilis* WI1) the 1.4 kb PCR amplicon obtained wit the Tn22 and 86CTn2 primers, was sequenced as previously described (GenBank Acc. No. KC311790) [18]. Statistical analysis was carried out with the SigmaPlot (Systat Software, Inc., Erkrath, Germany) software.

3. Results and discussion

Among the 486 *B. fragilis* strains studied, 68 were *bft*-positive (14.0%). From the PCR RFLP analysis, it was learned that 51 (75.0%) carried the *bft1* allele, 15 (22.1%) carried the *bft2* allele and 2 (2.9%) carried the *bft3* allele. To explore the roles of these alleles in non-intestinal pathogenesis, we checked the distribution of the three *bft* types in different clinical samples, especially among blood culture isolates. Some researchers detected different occurrences of ETBF strains from blood cultures, but these differences were not statistically significant [19,20]. The overall *bft* prevalence or the prevalences of the *bft1–3* alleles among the strains examined in our study were not significantly elevated among the blood culture isolates (9.2% vs. 7.3%), but the number of isolates obtained from blood cultures was low ($n = 5$), which could be the cause of the negative test result.

Of the 486 *B. fragilis* strains, 43 were *cfiA*-positive which were reported earlier together with the explored and detailed imipenem resistance mechanisms [17]. Surprisingly, of the 68 *bft*-positive *B. fragilis* strains, 4 were also *cfiA*-positive (Table 1). Comparison of prevalences of the *bft* genes among the *cfiA*-negative (14.4%) and *cfiA*-positive (9.3%) strains by using Fischer's exact test did not reveal a statistically significant difference, which means a basically equal distribution of the *bft* genes among *cfiA*-positive and -negative strains. The strains originated from different geographical locations and from various serious infection sites (Table 1). The

Table 1
Characteristics of the *bft–cfiA* doubly positive *B. fragilis* strains.

Characteristics detected by PCR (primers)	*B. fragilis*				
	WI1 (R19811) (UK, blood[e])	TR6 (Turkey, intra-abdominal abscess[e])	BEB15 (Belgium, wound[e])	IT9 (Italy[e])	HU92 (Hungary, periproctal abscess[e])
cfiA (cfiA1 and cfiA2)[a]	–	+	+	+	+
Imipenem MICs (μg/ml)	≥256	0.5	2	2	8
cfiA upstream (G and UP2)[a]	IS*614*[f]	Silent	Silent	Silent	Silent
bft gene (PCR RFLP) (BBT1 and BTT2)[b]	*bft1*	*bft1*	*bft1*	*bft1*	*bft1*
Presence of the 7-kb region of CTn9343 (Tn*25*B and Tn*25*C)[c]	–[g]	–	–	–	–
Lack of the 7-kb region of CTn9343 (Tn*25*B and Tn*9*B)[c]	–	–	–	–	–
Left end of CTn*86* (86CTn1R and Tn*9*B)[c]	1.9[h]	1.9	1.9	1.9	1.9
Insertion site of BfPAI into CTn*86* (P1T7 and P1T3)[c]	–	–	–	–	–
Left end of BfPAI in CTn*86* (P1T3 and P1T7-1)[c]	1.4	1.4	1.4	1.4	1.4
Right end of BfPAI in CTn*86* (P1T3 and P1T3-1)[c]	1.3	1.3	1.3	1.3	1.3
Excised circular form of CTn*86* (Tn*22* and 86CTn2)[d]	1.4[b]	1.4	1.4	1.4	1.4

[a] According to our previous studies.
[b] See in Methods section.
[c] According to Buckwold et al. and Franco [4,5].
[d] According to Buckwold et al. and Franco [4,5].
[e] Geographical origin and source of the strains as available from our database.
[f] Data taken from Terhes et al. [16].
[g] – indicates the absence of a product, the sizes of the positive PCR products being given in kb.
[h] The PCR product of *B. fragilis* WI1 (R19811) was sequenced.

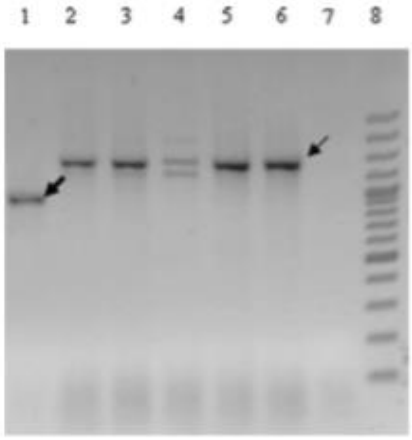


Fig. 1. PCR detection of the excised circular forms of the CTn*86* conjugative transposons in the *cfiA–bft* doubly positive *B. fragilis* strains. Lane 1: *B. fragilis* ATCC25285 (Tn*22* and Tn*25A* primers) [6]; lanes 2–7 *B. fragilis* TR6, IT9, BEB15, HU92, WI1 (R19811) and ATCC25285 (Tn*22* and 86CTn*2* primers); lane 8: molecular marker bands (GeneRuler 100 bp DNA Ladder Plus, Fermentas, Vilnius, Lithuania). The PCR products of the closed ends of CTn*9343* and CTn*86* are indicated by thick and thin arrows, respectively.

'silent' ($n = 4$, 80%) and 'expressed' ($n = 1$, 20%) antibiotic resistance mechanisms for imipenem were also represented roughly equally in the *cfiA–bft* doubly positive *B. fragilis* strains (9.3%) as among other Division II *B. fragilis* strains (overally ca. 8–12% of which is 'silent' and is phenotypically resistant) [10,17,21–24].

We were also interested in the molecular background of the *cfiA–bft* doubly positive strains because we wished to learn more about the factors that facilitate the emergence of such strains, which were earlier considered non-existent or very exceptional. Accordingly besides, the imipenem resistance mechanisms, we characterized the CTn*86* types in these strains and the previous *B. fragilis* WI1 strain. The molecular data acquired are listed in Table 1. From the 8 different molecular characteristics examined for the CTn*86* elements, including the allele type of the *bft* genes, variations in the transposon structure and the detection of the excised circular form of the CTns, all displayed similar results for the 5 *B. fragilis* strains studied (Table 1). All 5 *B. fragilis* strains carried the *bft1* allele, which was most frequently observed in other studies in Western populations [19,20,25–27]. CTn*86* has molecular properties similar to those found in the genomic sequence of *B. fragilis* NCTC9343 [5]. CTn*86* and CTn*9343* differ as concerns (i) their left end, (ii) CTn*86* may carry BfPAI, a 6 kb element harboring the *bft* genes, and (iii) CTn*9343* may carry an additional 7 kb element [5,6]. Two of these variants are sometimes found in a single *B. fragilis* strain, and, depending on the properties of these CTns detected by PCR patterning, the *B. fragilis* strains can be placed into 7 pattern groups (I.1–3., II. and III.1–3.). To detect CTn*86* with more certainty, we PCR-amplified the joined ends of the excised and circularized CTn*86*s from the doubly positive strains (Fig. 1). We could detect the excised closed ends of all the CTn*86*s of all the five strains examined. However *B. fragilis* BEB15 showed less specificity since the specific (1.4 kb, Fig. 1) band was fainter and some by-product bands strenghthened that were present in little quantity in two other reactions (*B. fragilis* TR6 IT9, Fig. 1). Additionally, we determined the nucleotide sequence of the excision product from *B. fragilis* WI1 (GenBank acc. No. KC311790). The obtained sequence displayed a structure similar to that from *B. fragilis* 86-5443-2-2 in the study by Franco [5]; *tnpA1* and *tnpB* ORFs at the left end and *tnpA2* ORFs at the right end, and two four-basepair inverted repeats (TACA and TGTA) bordering a TC dinucleotide. The left and right end sequences were 99 and 100% identical to the sequences from *B. fragilis* 86-5443-2-2 despite that *B. fragilis* 86-5443-2-2 harbors a *bft2* allele.

Since our strains belonged to the most prevalent *bft1* type and the most prevalent ETBF pattern (I.1, ca. 75%), we inferred that the most common CTn*86* elements appear to be most often detected in Division II *B. fragilis* strains too.

From these two groups of results, i.e. the equal frequencies of occurrences of the *bft* genes among *cfiA*-negative and -positive *B. fragilis* strains and the molecular properties, we conclude that no additional apparent requirements regarding the carrying conjugative transposons are needed for such doubly positive strains to occur. We also infer that the *bft* gene-carrying conjugative transposons can readily enter Division II *B. fragilis* strains, and our previous inability to detect them was due to the low number of isolates tested. Our present findings confirm the expected *in vivo* mobile property of the *bft* conjugative transposon, CTn*86*. The mobile property is consistent with the fact that no clonality was observed among the *bft*-positive strains in earlier studies [12,13,28]. The known *cfiA–bft1* double positive *B. fragilis* strains were from distant geographic sites indicating independent transfer events. A recent report also presented the transferable nature of the *bft* genes and possibly the carrying CTns since *bft* genes were detected among non-*fragilis Bacteroides* species from the microbiota of patients with colorectal carcinomas [29]. This latter finding further substantiates the mobile nature of the *bft* genes, and warrants additional research involving the molecular biology of the *bft* gene-carrying *Bacteroides* isolates.

In summary, this study has revealed the unexpected occurrence of *cfiA–bft* doubly positive strains, which we also characterized by molecular methods, especially with respect to the *bft*-carrying CTns. This finding is explained by the anticipated transferable property of the CTn*86 bft* gene-carrying genetic elements.

Acknowledgment

This study was supported by the Excellence Center of University of Szeged (TÁMOP 421B), the Hungarian National Research Fund (OTKA 69044) and the ESCMID Study Group of Anaerobic Infections.

References

[1] McGowan K, Gorbach SL. Anaerobes in mixed infections. J Infect Dis 1981;144:181–6.
[2] Wexler HM. Bacteroides: the good, the bad and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.
[3] Sears CL. Enterotoxigenic *Bacteroides fragilis*: a rogue among symbiotes. Clin Microbiol Rev 2009;22:349–69.
[4] Franco AA, Cheng RK, Chung G-T, Wu S, Oh H-B, Sears CL. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. J Bacteriol 1999;181:6623–33.
[5] Franco AA. The *Bacteroides fragilis* pathogenicity island is contained in a putative novel conjugative transposon. J Bacteriol 2004;186:6077–92.
[6] Buckwold SL, Shoemaker NB, Sears CL, Franco AA. Identification and characterization of conjugative transposons CTn*86* and CTn*9343* in *Bacteroides fragilis* strains. Appl Environ Microbiol 2007;73:53–63.
[7] Moncrief JS, Duncan AJ, Wright RL, Barroso LA, Wilkins TD. Molecular characterization of the *fragilis* in pathogenicity islet of enterotoxinogenic *Bacteroides fragilis*. Infect Immun 1998;66:1735–9.
[8] Nagy E. Anaerobic infections: update on treatment considerations. Drugs 2010;70:841–58.
[9] Johnson JL. Taxonomy of *Bacteroides* I. Deoxyribonucleic acid homologies among *Bacteroides fragilis* and other saccharolytitic *Bacteroides* species. Int J Sys Bacteriol 1978;28:245–56.
[10] Podglajen I, Breuil J, Casin I, Collatz E. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR-

generated fragment patterns and insertion sequence content. J Bacteriol 1995;177:5270–5.
[11] Ruimy R, Podglajen I, Breuil J, Christen R, Collatz E. A recent fixation of *cfiA* genes in a monophyletic cluster of *Bacteroides fragilis* is correlated with the presence of multiple insertion elements. J Bacteriol 1996;178:1914–8.
[12] Gutacker M, Valsangiacomo C, Piffaretti JC. Identification of two genetic groups in *Bacteroides fragilis* by multilocus enzyme electrophoresis: distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes. Microbiology 2000;146:1241–54.
[13] Gutacker M, Valsangiacomo C, Bernasconi MV, Piffaretti J-C. *recA* and *gltA* sequences separate the *Bacteroides fragilis* population into two genetic divisions associated with the antibiotic resistance genotypes *cepA* and *cfiA*. J Med Microbiol 2002;51:123–30.
[14] Wybo I, De Bel A, Soetens O, Echahidi F, Vandoorslaer K, Van Cauwenbergh M, et al. Differentiation of *cfiA*-negative and *cfiA*-positive *Bacteroides fragilis* isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2011;49:1961–4.
[15] Nagy E, Becker S, Sóki J, Urbán E, Kostrzewa M. Differentiation of division I (*cfiA*-negative) and division II (*cfiA*-positive) *Bacteroides fragilis* strains by matrix-assisted laser desorption/ionization time of flight mass spectrometry. J Med Microbiol 2011;60:1584–90.
[16] Terhes G, Ilrazier JS, Sóki J, Urbán E, Nagy E. Coincidence of *bft* and *cfiA* genes in a multi-resistant clinical isolate of *Bacteroides fragilis*. J Med Microbiol 2007;56:1416–8.
[17] Sóki J, Eitel Z, Urbán E, Nagy E. Molecular analysis of the carbapenem and metronidazole resistance mechanisms of *Bacteroides* strains reported in a Europe-wide antibiotic resistance survey. Int J Antimicrob Agents 2013;41:122–5.
[18] Sóki J, Gonzalez SM, Urbán E, Nagy E, Ayala JA. Molecular analysis of the effector mechanisms of cefoxitin resistance among *Bacteroides* strains. J Antimicrob Chemother 2011;66:2492–500.
[19] Claros MC, Claros ZC, Tang YJ, Cohen SH, Silva J, Goldstein EJC, et al. Occurrence of *Bacteroides fragilis* enterotoxin gene-carrying strains in Germany and the United States. J Clin Microbiol 2000;38:1996–7.
[20] Claros MC, Claros ZC, Hecht DW, Citron DM, Goldstein EJC, Silva Jr J, et al. Characterization of the *Bacteroides fragilis* pathogenicity island in human blood culture isolates. Anaerobe 2006;12:17–22.
[21] Edwards R, Hawkyard CV, Garvey MT, Greenwood D. Prevalence and degree of expression of the carbapenemase gene (*cfiA*) among clinical isolates of *Bacteroides fragilis* in Nottingham, UK. J Antimicrob Chemother 1999;43:73–276.
[22] Yamazoe K, Kato N, Kato H, Tanaka K, Katagiri Y, Watanabe K. Distribution of the *cfiA* gene among Bacteroides *fragilis* strains in Japan and relatedness of *cfiA* to imipenem resistance. Antimicrob Agents Chemother 1999;43:2808–10.
[23] Roh KH, Kim S, Kim CK, Yum JH, Kim MS, Yong D, et al. New *cfiA* variant and novel insertion sequence elements in carbapenem-resistant *Bacteroides fragilis* isolates from Korea. Diagn Microbiol Infect Dis 2010;66:343–8.
[24] Soki J, Urban E, Szilke I, Fodor E, Nagy E. Prevalence of the carbapenemase gene (*cfiA*) among clinical and normal flora isolates of *Bacteroides* species in Hungary. J Med Microbiol 2000;49:427–30.
[25] d'Abusco AS, Del Grosso M, Censini S, Covacci A, Pantosti A. The alleles of the *bft* gene are distributed differently among enterotoxigenic *Bacteroides fragilis* strains from human sources and can be present in double copies. J Clin Microbiol 2000;38:607–12.
[26] Pantosti A, Malpeli M, Wilks M, Menozzi MG, D'Ambrosio F. Detection of enterotoxigenic *Bacteroides fragilis* by PCR. J Clin Microbiol 1997;35:2482–6.
[27] Avila-Campos MJ, Liu C, Song Y, Rowlinson M-C, Finegold SM. Determination of *bft* gene subtypes in *Bacteroides fragilis* clinical isolates. J Clin Microbiol 2007;45:1336–8.
[28] Smith CJ, Callihan DR. Analysis of rRNA restriction fragment length polymorphisms from *Bacteroides* spp. and *Bacteroides fragilis* isolates associated with diarrhea in humans and animals. J Clin Microbiol 1992;30:806–12.
[29] Goodwin A, Badani R, Hiechenbleiker E, Chen L, Ellis B, Romans-Judge K, et al. Isolation of novel, non-*Bacteroides fragilis* strains containing the *Bacteroides fragilis* toxin (*bft*) locus from colorectal cancer patients. In: The 11th biennial congress of the Anaerobe Society of the Americas, San Francisco, CA, USA 2012SV-2.

PAPER III

Anaerobe 21 (2013) 43–49

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular biology, genetics and biotechnology

The prevalence of antibiotic resistance genes in *Bacteroides fragilis* group strains isolated in different European countries

Zsuzsa Eitel, József Sóki*, Edit Urbán, Elisabeth Nagy on behalf of ESCMID Study Group on Anaerobic Infection[1]

Institute of Clinical Microbiology, University of Szeged, Semmelweis u. 6, H-6725 Szeged, Hungary

ARTICLE INFO

Article history:
Received 8 November 2012
Received in revised form
21 February 2013
Accepted 5 March 2013
Available online 14 March 2013

Keywords:
Antibiotics
Antibiotic resistance
Antibiotic resistance genes
Bacteroides
Genetic elements

ABSTRACT

From the 2008–2009 European *Bacteroides* antibiotic resistance survey, we selected 161 strains for detection of antibiotic resistance genes (*cepA*, *cfxA*, *cfiA*, *nim*, *ermB*, *ermF*, *ermG*, *linA*, *mefA*, *msrSA*, *tetM*, *tetQ*, *tetX*, *tetX1*, *tet36* and *bexA*). To facilitate the throughput, the genes were detected by Real-Time PCR. The presence of the genes was correlated with the known MIC data of the strains for the appropriate antibiotics. For the β-lactams, the *cepA* gene was found in 70.8% of the tested strains (all resistant to ampicillin), but its presence did not correlate with the ampicillin MIC values. The *cepA* gene occurred at different frequencies among *Bacteroides fragilis* and non-fragilis *Bacteroides* strains. The *cfxA* gene was not a major factor in determining cefoxitin resistance and it was found with higher prevalence in non-fragilis *Bacteroides* strains than in *B. fragilis*. Among the five possible clindamycin resistance genes, *ermF* was the most common and had the highest effect on clindamycin resistance after *linA*. The *ermG-mefA-msrSA* combination was found in a set of strains and their linked occurrence implied that they were harbored by the conjugative transposon CTnGERM1. All strains tested were susceptible to metronidazole and none of them harbored *nim* genes. *TetQ* was prevalent among both the *B. fragilis* and non-fragilis *Bacteroides* strains (78.9 and 84.8%, respectively) and no gene could be clearly linked to tigecycline resistance other than *tetQ*. *BexA*, which codes for the fluoroquinolone efflux pump, was found in 7.5% of strains and occurred at different frequencies among *B. fragilis* and non-fragilis *Bacteroides* strains, but was represented only in a minor proportion of moxifloxacin-resistant strains.

1. Introduction

Anaerobic bacteria, including the most frequently isolated *Bacteroides fragilis* group strains, are members of the normal, resident gastrointestinal microbiota. They also colonize the oral cavity, upper respiratory tract and the female genital tract [1,2]. *Bacteroides* strains are abundant commensal anaerobic bacteria in the colon, but they are also human opportunistic pathogens that cause severe intra-abdominal infections, postoperative wound, skin and soft-tissue infections, together with other anaerobic and aerobic bacteria. They have been also identified as the causative agents of bacteremias [1]. An increasing resistance to antibiotics has been reported in *B. fragilis* group strains in recent years, and this has also been observed both in European countries and the United States [3,4]. Horizontal spread of antibiotic resistance among *B. fragilis* group clinical isolates is due to antibiotic resistance genes carried on conjugative and mobilizable plasmids, conjugative transposons and integrated genetic elements [5].

β-lactamase production is one of the most important mechanisms of resistance to β-lactam antibiotics in *B. fragilis* group strains. The *cepA* gene encodes the β-lactamase protein, which successfully hydrolyzes most of the cephalosporins (except cefoxitin) and penicillins. Resistance to cefoxitin has been shown to be associated with differences among the cefoxitin binding affinities of penicillin-binding proteins, and the presence of the *cfxA* gene, detected on mobilizable transposon, MTn*4555*, which is able to spread by conjugation between *Bacteroides* strains [5]. Resistance to carbapenems has been shown to be caused by the presence of the *cfiA* gene that is located on the bacterial chromosome. The *cfiA* gene may be expressed to various degrees or may be silent, causing different levels of carbapenem resistance. The insertion of an IS element may be responsible for the increased expression of the *cfiA* gene product [6].

* Corresponding author. Tel.: +36 62 545399; fax: +36 62 545712.
E-mail addresses: soki.jozsef@med.u-szeged.hu, dr.soki.jozsef@invitel.hu (J. Sóki).

[1] Denis Piérard (Belgium), Smilja Kalenić (Croatia), Eva Chmelařova (Czech Republic), Luc Dubreuil (France), Erika Dósa (Germany), Joseph Papaparaskevas (Greece), Cecilia Miszti (Hungary), Maria Hedberg (Sweden), Nurver Ulger (Turkey).

1075-9964/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.anaerobe.2013.03.001

Metronidazole, a 5-nitroimidazole, is regarded as an important therapeutic agent in the treatment of anaerobic infections. Resistance to metronidazole has been shown to be associated with the presence of the nitroimidazole resistance gene, *nimA-G*, which may be either located on plasmids or on chromosomes [7–9]. Although the resistance to metronidazole among *Bacteroides* strains is fairly rare, recent data show an emergence of resistance in the *B. fragilis* group isolates against metronidazole, especially in South Africa [4,10], but for Europe we found a decline as regards the prevalences of the *nim* genes [20].

Clindamycin resistance is a growing problem in anaerobe infections because clindamycin is still a drug that is frequently chosen by clinicians. Clindamycin resistance in the *B. fragilis* group isolates is mainly mediated by a macrolide-lincomycin-streptogramin (MLS) mechanism (*ermB*, *ermF* and *ermG* genes), but the efflux-pump mechanism is also observed, such as *msrSA* and *mefA*-mediated macrolid-lincosamide resistance [11]. The *ermF* gene is often associated with the presence of the *tetQ* gene on tetracycline resistance conjugative transposons. Transferable plasmids that can cause clindamycin resistance are the pBF4 (41 kb), pBFTM10 (pCP1) (15 kb) and pB1136 (80 kb). The *ermF* resistance gene can be found on the following transposons (harbored on the plasmids in brackets): Tn*4351* (pBF4), Tn*4400* (pBFTM10) and Tn*4551* (pB1136). In addition, genes involved in clindamycin resistance may also be located on chromosomal conjugative transposons as well (*ermFU*). The *linA* gene, which is an O-nucleotidyltransferase and located in NBU2 (non-replicating *Bacteroides* unit, which are also mobilizable transposons), may also be responsible for clindamycin and lincomycin resistance [12]. The *msrSA* gene encodes for a cell-membrane protein that acts as an active erythromycin-efflux pump. This gene was first described in a *Staphylococcus aureus* isolate, but it was also found in *Bacteroides* strains [13,14].

TetQ, *tetM* and *tet36* encode proteins that protect the bacterial ribosomes from tetracycline [15]. *TetQ* gene-related resistance is frequently observed among the *B. fragilis* group isolates [16]. The *tet36* gene was not present in human clinical and intestinal *Bacteroides* isolates, but was found in diverse bacterial genera including *Bacteroides* taken from swine manure [17]. The product of *tetX* and *tetX1* genes is an FAD-dependent monooxygenase, which destroys tetracycline, but may also raise the MIC values of tigecycline [18]. The *tetX* gene occurs on Tn*4351* and Tn*4400* transposons and regions associated with *ermF* on conjugative *tetQ* elements.

Members of the *Bacteroides* genus were originally resistant to 1st and 2nd generation quinolones, but the 3rd and 4th generation quinolones are still effective antibiotics in the treatment of infections involving anaerobic bacteria; and resistance to them has only begun to appear in the last few years. BexA is an efflux pump, which is encoded by the *bexA* gene, a member of the multidrug and toxic compound extrusion class (MATE) and has been described in *Bacteroides thetaiotaomicron* [19]. This efflux pump is one of the possible resistance genes responsible for fluoroquinolone resistance, and elevated moxifloxacin MIC values in *B. fragilis* group strains.

The aim of the present study was to define the general occurrence of *cepA*, *cfxA*, *cfiA*, *nim*, *ermB*, *ermF*, *ermG*, *linA*, *mefA*, *msrSA*, *tetM*, *tetQ*, *tetX*, *tetX1*, *tet36* and *bexA* genes and determine the roles that each play in antibiotic resistance in a range of *B. fragilis* group strains isolated in different European countries.

2. Materials and methods

2.1. Bacterial strains and cultivation

The 161 *B. fragilis* group strains examined in our study was a subset of isolates collected for a European antibiotic resistance study in 2008–2009 [4]. All the strains were clinical isolates of samples taken from 9 European countries (Belgium, Croatia, Czech Republic, France, Germany, Greece, Hungary, Sweden and Turkey) and belonged to the following species: *B. fragilis* ($n = 128$) and non-fragilis *Bacteroides* (NFB), *Bacteroides vulgatus* ($n = 11$), *B. thetaiotaomicron* ($n = 8$), *Bacteroides ovatus* ($n = 8$), *Bacteroides eggerthii* ($n = 2$), *Bacteroides distasonis* ($n = 2$), *Parabacteroides merdae* ($n = 1$) and *Bacteroides uniformis* ($n = 1$) (Table 1). The isolates were stored in Brain Heart Infusion (BHI) broth with 15% glycerine at −80 °C. The MIC values of the nine antimicrobial agents (ampicillin, cefoxitin, clindamycin, amoxicillin/clavulanic acid, piperacillin/tazobactam, tigecycline, imipenem/cilastatin, metronidazole and moxifloxacin) were determined previously by the agar dilution technique, as recommended by the CLSI [4]. The strains were cultured on anaerobic agar plates (Brucella blood agar supplemented with 5% sheep blood, 10 mg/l hemin and 1 mg/l vitamin K1) and incubated at 37 °C in an anaerobic atmosphere (Concept 400 anaerobic incubator, Biotrace International Plc., UK) for 48 h. The *B. fragilis* 638R (*cepA*), *B. vulgatus* CLA341 (*cfxA* and *tetQ*), *B. fragilis* TAL3636 (*cfiA*), *B. fragilis* BF8 (*ermF* and *nimB*), *B. fragilis* 638R [pIP417] (*nimA*), *B. fragilis* 638R [pIP421] (*nimD*), *B. thetaiotaomicron* BT13 (*nimC*), *B. fragilis* 388 (*nimE*), *B. thetaiotaomicron* 4001 [pGERM] (*ermG*, *mefA* and *msrSA*) *B. fragilis* BM13 (*tetX1*), *B. fragilis* TR23 (*linA*), *Clostridium difficile* 630 (*tetM* and *ermB*) and *Escherichia coli* AG102AX [pBRT20] (*bexA*) and DH5α [pGW140.1] (*tet36*) were used as control strains in the Real-Time PCR assays to assess the reliability of the results obtained (plasmid names in brackets and the gene names in parenthesis).

2.2. Real-Time PCR (RT-PCR) detection of the resistance genes

Bacterial cells from the surface of anaerobic agar plates were suspended in 100 μl water in 1.5 ml Eppendorf tubes, and incubated at 100 °C for 10 min. The supernatants of the centrifuged suspensions (2 min, 14,000 rpm) were used as template DNA and stored at −20 °C. Primers suitable for providing products in RT-PCR experiments, using the known nucleotide sequences of the genes, were

Table 1
Distribution of the *Bacteroides* species investigated in this study, categorized according to the origin of the isolate.

Tested *B. fragilis* group strains	Countries								
	Belgium	Croatia	Czech Republic	France	Germany	Greece	Hungary	Sweden	Turkey
B. fragilis ($n = 128$)	19	11	14	4	18	4	41	5	12
B. vulgatus ($n = 11$)	1	4	0	0	0	1	3	0	2
B. thetaiotaomicron ($n = 8$)	1	3	0	0	0	0	0	3	1
B. ovatus ($n = 8$)	1	2	1	0	0	2	1	0	1
B. eggerthii ($n = 2$)	0	0	0	0	0	2	0	0	0
B. distasonis ($n = 2$)	0	0	0	0	0	1	1	0	0
B. merdae ($n = 1$)	1	0	0	0	0	0	0	0	0
B. uniformis ($n = 1$)	0	0	0	0	0	0	1	0	0
Altogether: 161	23	20	15	4	18	10	47	8	16

designed by the Primer3 software (http://frodo.wi.mit.edu/) (Table 2). Each reaction mixture contained a 5 µl 2x PCR "mastermix" (iQ, Bio-Rad or Brilliant II, Stratagene), 0.7 µl (35 pmoles) of each primer, 1 µl template DNA, 0.5 µl EvaGreen (Biotium) DNA-binding fluorescent dye (for the iQ "mastermix") dye and sterile water up to 10 µl final volumes in plastic PCR plates. Amplification was performed in MxPro3000 (Stratagene, USA) or StepOne (Life-Technologies) Real-Time PCR instruments. The amplification and the melting curve were observed at a wavelength of 415 nm required for the SYBR Green and the EVA Green dyes. The initial denaturation by the amplification cycles was 10 min (iQ) or 5 min (Brilliant II). The PCR conditions with the various genes, primer sequences and PCR parameters are given in Table 2. Nucleotide sequencing of the *tetX1* (*B. fragilis* BM13) and *linA* (*B. fragilis* TR23) was carried out as described previously [23], and their sequences were compared to the reference sequences (*linAn2* AF251288 and *tetX1* AJ311171) by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Statistical evaluation

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

3. Results and discussion

The aim of this study was to determine whether the presence of selected antibiotic resistance genes was related to the physiological resistance data obtained in the previous European *Bacteroides*

Table 2
Oligonucleotide primers and the parameters used for the detection of resistance genes, with Real-Time PCR analysis.

Gene	Primer 5′→3′	PCR cycles
cepA	TTTCTGCTATGTCCTGCCT ATCTTTCACGAAGACGGC	95 °C 15 s, 56 °C 30 s, 72 °C 1 min, 35×
cfiA	AATCGAAGGATGGGGTATGG CGGTCAGTGAATCGGTGAAT	94 °C 15 s, 59 °C 1 min, 72 °C 30 s, 40×
cfxA	TGACTGGCCCTGAATAATCT ACAAAAGATAGCGCAAATCC	95 °C 15 s, 55 °C 1 min, 72 °C 30 s, 35×
ermB	GCGGAATGCTTTCATCCTAA GCGTGTTTCATTGCTTGATG	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35×
ermF	TAGATATTGGGGCAGGCAAG GGAAATTGCGGAACTGCAAA	95 °C 15 s, 58 °C 1 min, 72 °C 30 s, 35×
ermG	ATAGGTGCAGGGAAAGGTCA TGGATTGTGGCTAGGAAATGT	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35×
linA	CTGGGGAGTGGATGTCTTGT AGTTGGCTTGTTTGGAAGTG	95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 35×
mefA	ATACCCCAGCACTCAATTCG CAATCACAGCACCCAATACG	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35×
msrSA	GGGAACTGAAAGATGGCAAA TACGAGCCTGTTTTCGCTTT	95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 35×
tetM	ATCCTTTCTGGGCTTCCATT TCCGTCACATTCCAACCATA	95 °C 15 s, 59 °C 30 s, 72 °C 30 s, 35×
tetQ	ATCGGTATCAATGAGTTGTT GACTGATTCTGGAGGAAGTA	95 °C 15 s, 50 °C 1 min, 72 °C 30 s, 35×
tetX	TTAGCCTTACCAATGGGTGT CAAATCTGCTGTTTCATTCG	95 °C 15 s, 55 °C 30 s, 72 °C 30 s, 35×
tetX1	TCAGGACAAGAAGCAATGAA TATTTCGGGGTTGTCAAACT	95 °C 15 s, 50 °C 1 min, 72 °C 30 s, 32×
tet36	TTTCTGGCAGAGGTAGAACG TTAATTCCTTGCCTTCAACG	95 °C 15 s, 57 °C 30 s, 72 °C 30 s, 35×
bexA	TAGTGGTTGCTGCGATTCTG TCAGCGTCTTGGTCTGTGTC	95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 35×
nim	ATGTTCAGAGAAATGCGGC GTAAGTG GCTTCCTCGCCTGTCACGTGCTC	94 °C 15 s, 62 °C 30 s, 72 °C 30 s, 35×

Table 3
Distribution of tested genes among *B. fragilis* group isolates taken from different European countries.

Countries (number of *B. fragilis* group strains)	Number of the strains harboring the resistance genes (%)															
	cepA	*cfiA*	*cfxA*	*nim*	*ermB*	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*	*tetM*	*tetQ*	*tetX*	*tetX1*	*tet36*	*bexA*
Belgium ($n = 23$)	13 (56.5)	11 (47.8)	3 (13.0)	0 (0.0)	0 (0.0)	7 (30.4)	1 (4.3)	7 (30.4)	4 (17.4)	1 (4.3)	0 (0.0)	21 (91.3)	3 (13.0)	1 (4.3)	0 (0.0)	1 (4.3)
Croatia ($n = 20$)	14 (70.0)	3 (15.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (25.0)	0 (0.0)	3 (15.0)	0 (0.0)	0 (0.0)	0 (0.0)	16 (80.0)	1 (5.0)	1 (5.0)	0 (0.0)	2 (10.0)
Czech Republic ($n = 15$)	12 (80.0)	1 (6.7)	1 (6.7)	0 (0.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (20.0)	3 (20.0)	0 (0.0)	0 (0.0)	9 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
France ($n = 4$)	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	2 (50.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)
Germany ($n = 18$)	15 (83.3)	2 (11.1)	1 (5.6)	0 (0.0)	0 (0.0)	3 (16.7)	0 (0.0)	3 (16.7)	2 (11.1)	0 (0.0)	0 (0.0)	16 (88.9)	1 (5.6)	0 (0.0)	0 (0.0)	1 (5.6)
Greece ($n = 10$)	5 (50.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	3 (30.0)	0 (0.0)	2 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (80.0)	2 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hungary ($n = 47$)	37 (77.1)	7 (14.6)	4 (8.3)	0 (0.0)	0 (0.0)	7 (14.6)	3 (6.4)	12 (25.0)	4 (8.3)	3 (6.3)	0 (0.0)	37 (77.1)	4 (8.3)	1 (2.1)	0 (0.0)	4 (8.3)
Sweden ($n = 8$)	3 (37.5)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (62.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (25.0)
Turkey ($n = 16$)	10 (62.5)	5 (31.3)	1 (6.3)	0 (0.0)	0 (0.0)	9 (56.3)	5 (31.3)	4 (25.0)	7 (43.8)	5 (31.3)	0 (0.0)	14 (87.5)	4 (25.0)	4 (25.0)	0 (0.0)	2 (12.5)
Altogether: 161	113 (70.2)	29 (18.0)	12 (7.5)	0 (0.0)	1 (0.6)	39 (24.2)	9 (5.6)	35 (21.7)	20 (12.4)	9 (5.6)	0 (0.0)	129 (80.1)	16 (9.9)	7 (4.3)	0 (0.0)	12 (7.5)

Table 4
Distribution of the cepA, cfxA, and cfiA genes among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of the genes with the *Bacteroides* strains resistance to ampicillin, cefoxitin and imipenem.

Bacteroides strains	Number of strains with resistance genes (%)		
	cepA	*cfxA*	*cfiA*
B. fragilis (n = 128)	101 (78.9)[b]	19 (14.8)[c]	12 (9.4)
Non-fragilis *Bacteroides* (n = 33)	12 (36.4)[b]	10 (30.3)[c]	0 (0.0)

Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistant strains with resistance genes (%)		
		cepA	*cfxA*	*cfiA*
B. fragilis (n = 128)	Ampicillin	101 (78.9)	–	–
B. fragilis (n = 11)	Cefoxitin	–	3 (27.3)	–
B. fragilis (n = 1)	Imipenem	–	–	1 (100.0)
Non-fragilis *Bacteroides* (n = 33)	Ampicillin	12 (36.4)	–	–
Non-fragilis *Bacteroides* (n = 9)	Cefoxitin	–	1 (11.1)	–
Non-fragilis *Bacteroides* (n = 0)	Imipenem	–	–	0 (0.0)

Ampicillin ≥ 2 μg/ml Cefoxitin ≥ 64 μg/ml Imipenem ≥ 16 μg/ml.
[a] Resistance breakpoints according to CLSI (MIC μg/ml).
[b] The difference is statistically significant ($p < 0.001$) between values marked by the superscript b letters.
[c] The difference is statistically significant ($p = 0.039$) between values marked by the superscript c letters.

antibiotic resistance survey [4]. Previously, 640 *Bacteroides* strains were screened for the *cfiA* and *nim* genes [20]. In this study, a molecular analysis was performed to learn more about the incidence of the frequently detected resistance genes among *B. fragilis* group strains. Out of the 640 strains, we chose 161 (128 *B. fragilis* and 33 NFB) strains in order to detect the occurrence of the following genes: *cepA*, *cfxA*, *cfiA*, *nim*, *ermB*, *ermF*, *ermG*, *linA*, *mefA*, *msrSA*, *tetM*, *tetQ*, *tetX*, *tetX1*, *tet36* and *bexA*. The selection criteria chosen were intended to represent the whole collection and to represent in particular one country, Hungary. The distribution of the genes among the strains by countries is shown in Table 3. The numbers of strains with certain gene combinations are shown in Table S1.

3.1. Distribution of resistance genes responsible for β-lactam resistance

All the *B. fragilis* strains (128) were resistant to ampicillin (MIC ≥ 2 μg/ml) and 101 of them (78.9%) harbored the *cepA* gene. Among the 33 NFB strains which were also resistant to ampicillin, only 12 of them (36.4%), carried the *cepA* gene (Table 4). The *cepA* gene was first observed in a high-level cephalosporinase producer *Bacteroides* strain (*B. fragilis* CS30) and was found to be frequent among the *B. fragilis* isolates [21,22]. In addition, the *cepA* gene is found in a subgroup of the *B. fragilis* population called Division I. The *cepA* gene distributed with different frequencies among *B. fragilis* and NFB strains significantly ($p < 0.001$, Table 4). The presence of the *cepA* gene did not correlate with the ampicillin MIC values of the tested strains (Fig. 1); rather, it occurred among all ampicillin MIC ranges (from 2 to 256 μg/ml). Hence we expect that besides the *cepA* gene, the role of other resistance mechanisms, such as affinities of penicillin-binding proteins or efflux mechanisms may be responsible for the ampicillin resistance [23]. The *cepA* gene had approximately similar distributions among isolates from different countries (Table 3).

Out of the 11 cefoxitin-resistant *B. fragilis* strains, 3 of them (27.3%) harbored the *cfxA* gene and out of the 9 resistant NFB strains just 1 (11.1%) harbored the *cfxA* gene (Table 4). The relationship between the cefoxitin MIC values of the strains and the carrying of the *cfxA* gene is depicted in Fig. 2. Interestingly, among the strains with high MIC values (>64 μg/ml), the *cfxA* gene was often absent, a finding similar to that obtained in our previous study [23]. Also, in

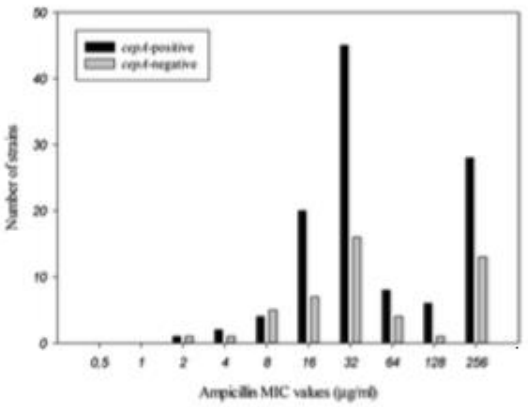


Fig. 1. The distribution of *cepA*-positive and *cepA*-negative *Bacteroides* strains in terms of ampicillin MIC values.

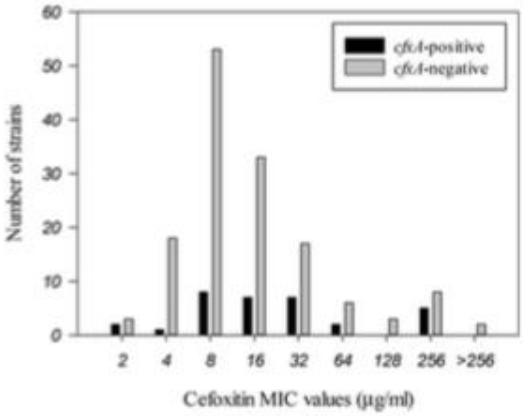


Fig. 2. The distribution of *cfxA*-positive and *cfxA*-negative *Bacteroides* strains in terms of the cefoxitin MIC values.

contrast to *cepA*, this gene was more common among NFB strains ($p = 0.039$, Table 4).

The *cfiA* gene was present in 12 (9.4%) *B. fragilis* strains, but only 1 of them was resistant to imipenem (MIC 32 μg/ml) (*B. fragilis* HU 61, Table 4). All 12 *cfiA*-positive isolates, including the strain HU 61, did not harbor any activating IS elements in the upstream regions of the *cfiA* genes. This phenomenon was observed in our previous study, where there were five *cfiA*-positive imipenem-resistant *B. fragilis* isolates that harbored no IS insertion upstream of the *cfiA* gene [20,24]. The overall prevalence of the *cfiA* gene among *B. fragilis* strains was 9.4% and the prevalence of the resistant strains was 0.8%, which is consistent with previous findings [25–29]. According to the literature, *cfiA*-positive NFB strains have not yet been detected.

3.2. Detection of resistance genes responsible for macrolide, lincosamide, streptogramin resistance

Of the 161 *Bacteroides* strains tested, 40 of them (24.8%) were resistant (MIC ≥ 8 μg/ml) to clindamycin. These consisted of 31 (24.2%) *B. fragilis* and 9 (27.3%) NFB isolates. The prevalence of the *ermF*, *linA*, *mefA*, *ermG*, *msrSA* and *ermB* genes among *Bacteroides* strains were 39 (24.2%), 35 (21.7%), 20 (12.4%), 9 (5.6%), 9 (5.6%) and 1 (0.6%), respectively (Table 5). The prevalence of the *ermF*, *linA*, *mefA*, *ermG*, *msrSA* and *ermB* resistance genes among the clindamycin-resistant *Bacteroides* strains were 30 (75.0%), 14 (35.0%), 11 (27.5%), 9 (22.5%), 9 (22.5%) and 1 (2.5%), respectively. The distribution of the clindamycin resistance genes (*ermB*, *ermF*, *ermG*, *linA*, *mefA* and *msrSA*) alone or in combination with other genes in *B. fragilis* or NBF strains among the tested isolates or in clindamycin-resistant strains is shown in Table 5 and also in Table S2. The most common resistance gene was *ermF*, accounting for most of the clindamycin resistant strains. The *ermF* gene was present in 23 (74.2%) of the 31 clindamycin-resistant *B. fragilis* strains tested and 7 (77.8%) of the 9 clindamycin-resistant NFB isolates tested. The incidence of the *ermF* gene was almost identical in the *B. fragilis* and NFB strains, regardless of whether they were resistant to clindamycin or not.

The *msrSA*-positive and the *ermG*-positive isolates harbored at least one other resistance gene and some isolates simultaneously harbored several types of clindamycin resistance genes (Table S2). For example, 6 *B. fragilis* strains (1 Belgian and 5 Turkish) contained the *ermF*, *ermG*, *msrSA* and *mefA* gene combination, all of which were resistant to clindamycin (Table S2). There were 9 *B. fragilis* isolates that carried the *ermG*, *mefA* and the *msrSA* genes together or harbored other clindamycin resistance genes. Presumably, strains like the *ermG*, *mefA* and *msrSA*-positive strains contained a conjugative transposon, CTnGERM1, which was once found to harbor these genes [30]. The distribution of strains like these based on their country of origin were as follows: 5 from Turkey, 3 from Hungary and 1 from Belgium.

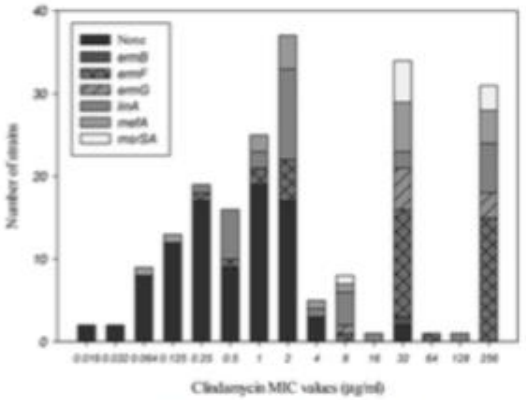


Fig. 3. Distribution of the clindamycin resistance genes across the Bacteroides strains with different clindamycin MIC values.

Summarizing the above findings, we may reasonably conclude that the clindamycin susceptible strains either did not harbor any gene or the fact that they may carry *ermF*, *linA* or *mefA* genes. The presence of *ermG* or the *msrSA* genes was not itself sufficient to cause resistance to clindamycin. Therefore, the main factor for clindamycin resistance might be the *ermF* gene alone or in combination with the CTnGERM1 genes (*ermG*, *mefA* and *msrSA*). The *linA* gene, probably residing on mobilizable transposons such as NBU2, was also a factor contributing to this type of resistance (Fig. 3, Table S2). The distribution of *mefA* was noticeably different ($p = 0.043$) among clindamycin-resistant *B. fragilis* and NFB strains (Table 5).

3.3. Prevalence of other resistance genes (tetM, tetQ, tetX, tetX1, tet36, nim, bexA) among B. fragilis and NFB strains

The prevalence of the *tetQ*, *tetX* and *tetX1* genes among 161 *Bacteroides* strains were 129 (80.1%), 16 (9.9%) and 8 (5.0%), respectively (Table 6). There were no *tetM*-positive or *tet36*-positive strain among the 161 *Bacteroides* isolates that were tested. Only 3

Table 5
Distribution of the *ermF*, *ermB*, *ermG*, *linA*, *mefA* and *msrSA* genes among the *B. fragilis* and non-fragilis Bacteroides strains and correlation of the genes with their resistance to clindamycin.

Bacteroides strains	Number of strains with resistance genes (%)					
	ermB	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*
B. fragilis ($n = 128$)	1 (0.8)	29 (22.7)	9 (7.0)[b]	28 (21.9)	17 (13.3)	9 (7.0)
Non-fragilis *Bacteroides* ($n = 33$)	0 (0.0)	10 (30.3)	0 (0.0)[b]	7 (21.2)	3 (9.1)	0 (0.0)
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistant strains with resistance genes (%)					
		ermB	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*
B. fragilis ($n = 31$)	clindamycin	1 (3.2)	23 (74.2)	9 (29.0)[c]	9 (29.0)[d]	11 (35.5)[e]	9 (29.0)
Non-fragilis *Bacteroides* ($n = 9$)		0 (0.0)	7 (77.8)	0(0.0)[c]	5 (55.6)[d]	0 (0.0)[e]	0 (0.0)

[a] Resistance breakpoint for clindamycin: ≥8 μg/ml according to CLSI.
[b,c] The differences between the prevalences are notable, but not statistically significant ($p = 0.117$ and $p = 0.09$, respectively) between values marked by the superscript b and c letters.
[d] The observed difference is not statistically significant ($p = 0.234$) between values marked by the superscript d letters, probably because of the low number of test strains.
[e] The difference is statistically significant ($p = 0.043$) between values marked by the superscript e letters.

Table 6
Distribution of the *tetM*, *tetQ*, *tetX*, *tetX1* and *tet36* genes among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of the genes with the resistance of the *Bacteroides* strains to tigecycline.

Bacteroides strains		Number of strains with resistance genes (%)				
		tetM	*tetQ*	*tetX*	*tetX1*	*tet36*
B. fragilis ($n = 128$)		0 (0.0)	101 (78.9)	13 (10.2)	7 (5.5)	0 (0.0)
Non-fragilis *Bacteroides* ($n = 33$)		0 (0.0)	28 (84.8)	3 (9.1)	1 (3.0)	0 (0.0)
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistant strains with resistance genes in them (%)				
		tetM	*tetQ*	*tetX*	*tetX1*	*tet36*
B. fragilis ($n = 3$)	Tigecycline	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Non-fragilis *Bacteroides* ($n = 0$)		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Resistance breakpoint for tigecycline: ≥16 μg/ml according to CLSI.

(1.9%) *B. fragilis* strains were resistant to tigecycline (MIC ≥ 16 μg/ml) and all of them carried the *tetQ* gene. Moreover, the *tetM*, *tetX*, *tetX1* and *tet36* genes were not present in any of the tigecycline-resistant *Bacteroides* strains.

In this study, all the strains tested were susceptible to metronidazole and none of them harbored the *nim* gene. In a previous study, we examined the prevalence of the *cfiA* and *nim* genes in a larger number of *Bacteroides* strains ($n = 640$) [20].

The *bexA* gene was present in 12 (7.5%) of the 161 *Bacteroides* isolates tested. These consisted of 6 (4.7%) *B. fragilis* strains and 6 (18.2%) NFB strains (Table 7). This difference is statistically significant ($p = 0.024$). There were no *bexA*-positive strains among the 18 moxifloxacin-resistant *B. fragilis* isolates, and of the 6 moxifloxacin-resistant NFB, only one (16.7%) harbored the *bexA* gene. It is possible that moxifloxacin resistance in the majority of the strains could be due to mutations in the *gyrA* gene which is the resistance mechanism most commonly found in fluoroquinolone resistant strains of other species [31,32]. However, the other resistance mechanisms should be confirmed by other experiments, e.g. sequencing of the *gyrA* genes, in the remaining moxifloxacin-resistant strains.

In this study we examined a set of antibiotic resistance genes that occurred among the *Bacteroides* strains found in Europe. In the case of certain genes (*cepA*, *cfxA*, *cfiA*), our data confirmed earlier findings: (i) *cepA* is very frequent among *Bacteroides* and can be found among NFB too [22,33,34], (ii) the prevalence of *cfxA* is around 15–20% and it is not an only factor for cefoxitin resistance [23,35,36], and (iii) *cfiA* has a frequency around 5–7% in *B. fragilis* strains [20,25,27–29,35]. The study also clarified the contribution of other genes (*ermF* and *tetQ*) or determined the prevalences of some recently described or unique genes (*tetX*, *tetX1*, *tet36*, *tetM*, *ermB*, *ermG*, *msrSA*, *mefA* and *linA*). The scale of the current study was greater than a smaller study reporting the detection, prevalence and combination of the most important resistance genes of *Bacteroides* and *Parabacteroides* spp. [33]. This latter work detected *cepA* (88.0%) and *tetQ* (48.0%) as the most frequent antibiotic resistance genes among a collection of clinical isolates of *B. fragilis* strains, and also found *cfxA* (12.0%), *cfiA* (4.0%), *ermF* (16.0%) and *nim* (4.0%) genes in this same collection. We detected the co-localization of some genes (*ermG*, *mefA* and *msrSA*) in a proportion of strains (most probably due to harboring CTnGERM1), however, we were not able to establish whether the genes in this combination were responsible for antibiotic drug resistance, but the results emphasize the prevalence of CTnGERM1. The above data might be useful in estimating the likelihood of emergence of the more resistant or multiple-resistant *Bacteroides* strains. Multiple antibiotic resistant *B. fragilis* strains pose a serious medical threat and in the past were often isolated in Europe [37]. In addition, since *Bacteroides* are important members of the normal intestinal microbiota, they are exposed to antibiotics used for various reasons by humans. They have also been observed as significant reservoirs and sources of antibiotic resistance genes. In our study, the *ermB* and *ermG* genes, which are characteristic for Gram-positive species [11], and the *tetX* and variant *tetX1* genes [18], which code for aerobic type tetracycline oxidizing enzymes, were found among *Bacteroides* isolates. Some of the resistance genes examined were, however, found in susceptible strains, which raise the question of their genetic regulation. It is well known that *cepA*, *cfxA*, *cfiA*, *nim* and the *ermF* genes are activated by insertion sequence elements in *Bacteroides* strains, which may be the case in the other genes as well.

The study reported here has provided a fairly complete picture concerning the antibiotic resistance levels and the antibiotic resistance genes responsible for their development in Europe. However, it could also be a starting point for the further investigation of the antibiotic resistance mechanisms of the *Bacteroides* species or for issues involving the prevalence of the antibiotic resistance genes isolated from the human intestinal microbiota.

Table 7
Distribution of the *bexA* gene among the *B. fragilis* and non-fragilis *Bacteroides* strains and correlation of their presence with the resistance of the *Bacteroides* strains to moxifloxacin.

Bacteroides strains		Number of strains with resistance genes (%)
		bexA
B. fragilis ($n = 128$)		6 (4.7)[b]
Non-fragilis *Bacteroides* ($n = 33$)		6 (18.2)[b]
Number of resistant *Bacteroides* strains[a]	Antibiotic	Number of resistance strains with resistance genes (%)
		bexA
B. fragilis ($n = 18$)	moxifloxacin	0 (0.0)[c]
Non-fragilis *Bacteroides* ($n = 6$)		1 (16.7)[c]

[a] Resistance breakpoint for moxifloxacin: ≥8 μg/ml according to CLSI.
[b] The differences between the prevalences are statistically significant ($p = 0.024$) between values marked by the superscript b letters.
[c] The observed difference is not statistically significant ($p = 0.250$) between values marked by the superscript c letters, probably because of the low number of test strains.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgments

This study was supported by a grant from ESGAI (part of ESCMID) given to Prof. Elisabeth Nagy and the Excellence Centre at the University of Szeged (TÁMOP-421B). We are grateful to Dr. Nadja Shoemaker, Dr. Patrizia Spigaglia and Dr. Hiroshi Nikaido for sending control materials (*E. coli* DH5α/pGW140.1 and AG102AX/pBRT20, *B. thetaiotaomicron* 4001/pGERM and *C. difficile* 630).

Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2013.03.001.

References

[1] Wexler HM. *Bacteroides*: the good, the bad and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.
[2] Finegold SM. Anaerobic bacteria in human disease. New York: Academic Press; 1977.
[3] Snydman DR, Jacobus NV, McDermott LA, Golan Y, Hecht DW, Goldstein EJC, et al. Lessons learned from the anaerobe survey: historical perspective and review of the most recent data (2005–2007). Clin Infect Dis 2010;50(Supplement 1):S26–33.
[4] Nagy E, Urbán E, Nord CE., On behalf of the ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. Clin Microbiol Infect 2011;17:371–9.
[5] Smith CJ, Tribble GD, Bayley DP. Genetic elements of *Bacteroides* species: a moving story. Plasmid 1998;40:12–29.
[6] Edwards R. Resistance to β-lactam antibiotics in *Bacteroides* spp. J Med Microbiol 1997;46:979–86.
[7] Haggoud A, Reysset G, Azeddoug H, Sebald M. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from *Bacteroides* strains and of a new insertion sequence upstream of the two genes. Antimicrob Agents Chemother 1994;38:1047–51.
[8] Reysset G, Haggoud A, Su W, Sebald M. Genetic and molecular analysis of pIP417 and pIP419: *Bacteroides* plasmids encoding 5-nitroimidazole resistance. Plasmid 1992;27:181–90.
[9] Reysset G. Genetics of 5-nitroimidazole resistance in *Bacteroides* species. Anaerobe 1996;2:59–69.
[10] Galvão BPGV, Meggersee RL, Abratt VR. Antibiotic resistance and adhesion potential of *Bacteroides fragilis* clinical isolates from Cape Town, South Africa. Aanerob 2011;17:142–6.
[11] Nakajima Y. Mechanisms of bacterial resistance to macrolid antibiotics. J Infect Chemother 1999;5:61–74.
[12] Wang J, Shoemaker N, Wang GR, Salyers AA. Characterization of a *Bacteroides* mobilizable transposon, NBU2, which carries a functional lincomycin resistance gene. J Bacteriol 2000;182:3559–71.
[13] Matsuoka M, Jánosi L, Endou K, Saitoh S, Hashimoto H, Nakajima Y. An increase of 63 kDa-protein present in the cell membranes of *Staphylococcus aureus* that bears a plasmid mediating inducible resistance to partial macrolide and streptogramin B antibiotics. Biol Pharm Bull 1993;16:1288–90.
[14] Cassone M, D'Andrea MM, Iannelli F, Oggioni MR, Ressolini GM, Pozzi G. DNA microarray for detection of macrolide resistance genes. Antimicrob Agents Chemother 2006;50:2038–41.
[15] Roberts MC. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol Rev 1996;19:1–24.
[16] Rasmussen BA, Bush K, Tally FP. Antimicrobial resistance in *Bacteroides*. Clin Infect Dis 1993;16:390–400.
[17] Whittle G, Whitehead TR, Hamburger N, Shoemaker NB, Cotta MA, Salyers AA. Identification of a new ribosomal protection type of tetracycline resistance gene, *tet*(36), from swine manure pits. Appl Environ Microbiol 2003;69:4151–8.
[18] Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl Environ Microbiol 2001;67:561–8.
[19] Miyamae S, Ueda O, Yoshimura F, Hwang J, Tanaka Y, Nikaido H. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides thetaiotaomicron*, Antimicrob. Agents Chemother 2001;45:3341–6.
[20] Sóki J, Eitel Zs, Urbán E, Nagy E., on behalf of the ESCMID Study Group on Anaerobic Infections. Molecular an'alysis of the carbapenem and metronidazole resistance mechanisms of *Bacteroides* strains reported in a Europe-wide antibiotic resistance survey. Int J Antimicrob Agents 2013;41:122–5.
[21] Rogers MB, Parker AC, Smith CJ. Cloning and characterization of the endogenous cephalosporinase gene, *cepA*, from *Bacteroides fragilis* reveals a new subgroup of Ambler Class A β-lactamases. Antimicrob Agents Chemother 1993;37:2391–400.
[22] Mastrantonio P, Cardines R, Spigaglia P. Oligonucleotide probes for detection of cephalosporinases among *Bacteroides* strains. Antimicrob Agents Chemother 1996;40:1014–6.
[23] Sóki J, Gonzalez SM, Urbán E, Nagy E, Ayala JA. Molecular analysis of the effector mechanisms of cefoxitin resistance among *Bacteroides* strains. J Antimicrob Chemother 2011;66:2492–500.
[24] Sóki J, Fodor E, Hecht DW, Edwards R, Rotimi VO, Kerekes I, et al. Molecular characterization of imipenem-resistant, *cfiA*-positive *Bacteroides fragilis* isolates from the USA, Hungary and Kuwait. J Med Microbiol 2004;53:413–9.
[25] Sóki J, Urban E, Szőke I, Fodor E, Nagy E. Prevalence of the carbapenemase gene (*cfiA*) among clinical and normal flora isolates of *Bacteroides* species in Hungary. J Med Microbiol 2000;49:427–30.
[26] Podglajen I, Breuil J, Casin I, Collatz E. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR-generated fragment patterns and insertion sequence content. J Bacteriol 1995;177:5270–5.
[27] Yamazoe K, Kato N, Kato H, Tanaka K, Katagiri Y, Watanabe K. Distribution of the *cfiA* gene among *Bacteroides fragilis* strains in Japan and relatedness of *cfiA* to imipenem resistance. Antimicrob Agents Chemother 1999;43:2808–10.
[28] Edwards R, Hawkyard CV, Garvey MT, Greenwood D. Prevalence and degree of expression of the carbapenemase gene (*cfiA*) among clinical isolates of *Bacteroides fragilis* in Nottingham, UK. J Antimicrob Chemother 1999;43:273–6.
[29] Roh KH, Kim S, Kim C-K, Yum JH, Kim MS, Yong D, et al. New *cfiA* variant and novel insertion sequence elements in carbapenem-resistant *Bacteroides fragilis* isolates from Korea. Diagn Microbiol Infect Dis 2010;66:343–8.
[30] Wang Y, Wang GR, Shelby A, Shoemaker NB, Salyers AA. A newly discovered *Bacteroides* conjugative transposon, CTnGERM1, contains genes also found in gram-positive bacteria. Appl Environ Microbiol 2003;69:4595–603.
[31] Oh H, El Amin N, Davies T, Appelbaum PC, Edlund C. *gyrA* mutations associated with quinolone resistance in *Bacteroides fragilis* group strains. Antimicrob Agents Chemother 2001;45:1977–81.
[32] Bachoual R, Dubreuil L, Soussy C-J, Tankovic J. Roles of *gyrA* mutations in resistance of clinical isolates and in vitro mutants of *Bacteroides fragilis* to the new fluoroquinolone trovafloxacin. Antimicrob Agents Chemother 2000;44:1842–5.
[33] Boente RF, Ferreira LQ, Falcão LS, Miranda KR, Guimarães PLS, Santos-Filho J, et al. Detection of resistance genes and susceptibility patterns in *Bacteroides* and *Parabacteroides* strains. Anaerobe 2010;16:190–4.
[34] Lorenzo M, García N, Ayala JA, Vadillo S, Píriz S, Quesada A. Antimicrobial resistance determinants among anaerobic bacteria isolated from footrot. Vet Microbiol 2012;157:112–8.
[35] Avelar KES, Otsuki K, Vicente ACP, Vieira JMBD, de Paula GR, Domingues RMCP, et al. Presence of the *cfxA* gene in *Bacteroides distasonis*. Res Microbiol 2003;154:369–74.
[36] García N, Gutiérrez G, Lorenzo M, García JE, Píriz S, Quesada A. Genetic determinants for *cfxA* expression inBacteroides strains isolated from human infections. J Antimicrob Chemother 2008;62:942–7.
[37] Harmeyer GN, Sóki J, Nagy E, Justesen US. Multidrug-resistant *Bacteroides fragilis* group on the rise in Europe? – a case report and review of the literature. J Med Microbiol 2012. [Epub ahead of print].

PAPER IV

Anaerobe xxx (2014) 1–4

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular Biology, Genetics and Biotechnology

Analysis of Romanian *Bacteroides* isolates for antibiotic resistance levels and the corresponding antibiotic resistance genes

Edit Székely [a, b], Zsuzsa Eitel [c], Szabolcs Molnár [a], Izabella Éva Szász [a], Doina Bilca [a], József Sóki [c, *]

[a] Diagnostic Laboratory, Emergency County Clinical Hospital, Gh. Marinescu 50, Targu-Mures, 540139, Romania
[b] Department of Microbiology, Faculty of Medicine, University of Medicine and Pharmacy of Targu Mures, Gh. Marinescu 38, Targu-Mures, 540139, Romania
[c] Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged, Semmelweis 6 H-6725 Szeged, Hungary

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Antibiotic susceptibility
Antibiotic resistance genes
Bacteroides

ABSTRACT

As part of an ESCMID Study Group on Anaerobic Infections (ESGAI) project, a study was conducted to measure the antibiotic susceptibilities and corresponding gene contents of 53 *Bacteroides fragilis* group strains isolated in Romania. The antibiotic resistance data was comparable with the data found for other East-European countries. Here, no resistant isolate was found for imipenem, metronidazole and tigecycline. An increasing role of the *cepA*, *cfxA* and *cfiA* genes was observed in their corresponding antibiotic resistances. Moreover, no isolate was found that harbored the *cfiA* gene with a possible activating IS element. Clindamycin resistance was low, similarly to that the rate for the ermF gene. However, we did find some isolates with *nimB*, *ermB*, *msrSA*, *linA*, *satG*, *tetX*, *tetM* and *bexA* genes. This study was the first to provide antibiotic resistance data for clinical *Bacteroides* strains from Romania.

1. Introduction

Anaerobic pathogens may cause severe infections and the most frequently isolated strains belong to *Bacteroides fragilis* and to some related species. These important species, are normally intestinal commensals that exert many useful interactions in the gut. In addition, clinical *Bacteroides* isolates have the highest antibiotic resistance levels and the highest number of antibiotic resistance mechanisms among all human pathogenic anaerobic bacteria. However, their special culturing requirements make their diagnostic processes and antimicrobial testing tedious and time consuming. Therefore regular geographical and temporal records of antimicrobial susceptibilities are essential for their empirical therapies. Regular resistance surveys have been conducted over the years in the USA [1], in Europe [2] and also in some other developed countries [3,4]. Thus the general picture that has emerged is that *Bacteroides* are almost completely resistant to 'regular β-lactams' (penicillins, cephalosporins) and tetracyclines, moderate resistance can be found for cefoxitin, amoxicillin/clavulanic acid, clindamycin and moxifloxacin, and the resistance is very low for carbapenems, piperacillin/tazobactam, metronidazole and tigecycline. The latest susceptibility survey for Europe has been completed with the identification of corresponding antibiotic resistance genes [5] along with a detailed antibiotic resistance mechanism analysis for metronidazole-resistant, *nim*-positive strains and strains with elevated imipenem resistance levels [6]. The particular antibiotic resistance genes that are suspected or proved to be behind the main antibiotic resistance phenotypes are as follows: *cepA* for 'regular β-lactams' (penicillins, cephalosporins), *cfxA* for cephamycins, *cfiA* for carbapenems, *ermF* for the MLSB (macrolide, lincosamide, streptogramin) group of antibiotics, *nim* genes for 5-nitroimidazole resistances, and *tetQ* for tetracyclines. Besides these, other less frequent antibiotic resistance genes may also be found among clinically important *Bacteroides* strains like *tetX* for tetracyclines and glycylcyclines, *msrSA* for streptogramins or *bexA* for fluoroquinolones. An interesting aspect of the antibiotic resistance mechanisms of *Bacteroides* is that special insertion sequence (IS) elements may activate the resistance genes to induce high resistance levels.

Romania did not send *Bacteroides* strains to the latest European survey, partly because anaerobic diagnostics is only performed to a limited extent. However, since 2008 at Targu-Mures (a university town in Transylvania) a good anaerobic diagnostics facility has been operating using good connections with the ESGAI and the National

* Corresponding author. Institute of Clinical Microbiology, Albert Szent-Györgyi Clinical Centre, Faculty of Medicine, University of Szeged, Semmelweis 6, H-6726 Szeged, Hungary. Tel.: +36 62 545399; fax: +36 62 545712.
E-mail addresses: soki.jozsef@med.u-szeged.hu, dr.soki.jozsef@digikabel.hu (J. Sóki).

http://dx.doi.org/10.1016/j.anaerobe.2014.09.001
1075-9964/

Table 1
Comparison of the susceptibility values detected in this study with those for Europe.

Group	Descripter	Ampicillin	Amoxicillin/clavulanic acid	Piperacillin/tazobactam	Cefoxitin	Clindamycin	Imipenem	Metronidazole	Moxifloxacin	Tetracycline	Tigecycline
European	Range	1–>256	0.016–>256	0.016–>256	1–>256	0.016–>256	0.002–>32	0.016–256	<0.125–32	–	0.016–32
Bacteroides	MIC_{50}	32	1	4	16	2	0.5	0.5	1	–	0.25
fragilis group	MIC_{90}	>256	16	32	128	>256	1	1	16	–	2
strains	Resistance (%)	98.2	10.4	10.3	17.2[a]	32.4	0.85[a]	0.5	13.6[a]	–	1.7[a]
(n = 824)											
Romanian	Range	1–>256	0.25–64	0.032–32	2–128	0.032–>256	0.032–2	0.125–1	0.125–>32	<0.5–256	0.032–8
Bacteroides	MIC_{50}	32	2	4	16	1	0.125	0.25	1	32	0.25
fragilis group	MIC_{90}	>256	16	16	64	4	1	0.5	>32	64	0.5
strains	Resistance (%)	96.3	13.0	5.6	14.8[a]	9.4	0	0	14.8[a]	75.9[a]	0[a]
(n = 53)											
European	Range	1–>256	0.016–>256	0.016–>256	1–>256	0.016–>256	0.002–>32	0.016–32	<0.125–64	–	0.016–32
Bacteroides	MIC_{50}	32	1	2	16	1	0.25	0.5	0.5	–	0.5
fragilis strains	MIC_{90}	>256	16	16	256	256	0.5	1	8	–	2
(n = 600)	Resistance (%)	97.4	8.7	6.5	13.7[a]	28.5	1.2	0.5	14.0[a]	–	1.8[a]
Romanian	Range	2–>256	0.25–64	0.032–32	4–64	0.064–>256	0.032–2	0.125–0.5	0.125–>32	<0.5–256	0.125–8
Bacteroides	MIC_{50}	32	2	2	16	1	0.125	0.25	1	32	0.25
fragilis strains	MIC_{90}	>256	16	8	32	2	1	0.5	>32	64	0.5
(n = 36)	Resistance (%)	97.3	13.5	2.7[b]	2.7[a,b]	2.8[b]	0	0	13.5[a]	73.0[a]	0[a]
Romanian	Range	1–>256	0.25–16	2–32	2–128	0.032–>256	0.064–1	0.25–1	0.5–>32	<0.5–64	0.032–2
non-fragilis	MIC_{50}	16	2	16	32	4	0.25	0.5	2	32	0.125
Bacteroides	MIC_{90}	>256	16	32	64	256	1	0.5	>32	64	1
strains	Resistance (%)	94.1	11.8	11.8[b]	41.2[a,b]	23.5[b]	0	0	17.6[a]	82.4[a]	0[a]
(n = 17)											

[a] Here, CLSI breakpoints could be applied.
[b] The significance values for differences between *B. fragilis* and non-fragilis *Bacteroides* strains were 0.238 for piperacillin/tazobactam, <0.001 for cefoxitin and 0.032 for clindamycin.

Reference Laboratory for Anaerobic Pathogens at Szeged. The *Bacteroides* strains collected between 2010 and 2013 were subjected to the same analyses as those done for other European countries in the latest susceptibility and antibiotic resistance mechanism study [2]. The main goal was to provide antibiotic resistance data for Romania, as this has not yet been reported for this European country.

2. Material and methods

2.1. Bacterial strains and cultivation

53 *B. fragilis* group strains (36 *B. fragilis*, 7 *Bacteroides thetaiotaomicron*, 7 *Bacteroides ovatus* and 3 *Bacteroides vulgatus*) were collected in the period 2010 and 2013 at the Diagnostic Laboratory of the Emergency County Clinical Hospital at Targu-Mures in Romania, taken a wide variety of clinical samples, which were mainly abdominal, wound and blood culture cases. The routine isolations and identifications were performed according to methods described in the literature [7]. The species identifications were positively confirmed using MALDI-TOF MS at the Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary. For long-term storage, 20% glycerol stocks were used at −70 °C. In further analyses the strains were cultivated at 37 °C anaerobically on Columbia agar supplemented with 5% (v/v) sheep blood, 5 g/L hemin and 1 g/L vitamin K_1, or in BHIS broth (brain-heart infusion broth supplemented with 0.5% (w/v) yeast extract, 5 g/L hemin and 1 g/L vitamin K_1), in anaerobic environment (85% N_2, 10% H_2, 5% CO_2) for 48 h.

2.2. Antibiotic susceptibility and genetic testing

Antibiotic susceptibility tests were carried out by agar dilution as described earlier for ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, imipenem, clindamycin, moxifloxacin, metronidazole, tetracycline and tigecycline [2]. The antibiotic resistance genes (*cepA*, *cfxA*, *cfiA*, *ermB*, *ermF*, *ermG*, *linA*, *mefA*, *msrSA*, *bexA,nim*, *tetM*, *tetQ*, *tetX*, *tetX1* and *tet36*) and one IS element (IS*4351*) were detected by RealTime PCR [5]. Chi-squared analyses were carried out using Sigmaplot 12.0 (Systat Software, Inc.) after setting the significance threshold level to 0.05.

3. Results and discussion

3.1. Antibiotic resistance levels

Of the 53 strains examined, MALDI-TOF MS was able to confirm or update (n = 5) the original identifications. Data for the antibiotic resistance levels obtained are displayed in Table 1, it is grouped into (i) all strains, (ii) *B. fragilis* and (iii) NFB (non-fragilis *Bacteroides*), and where available are compared with the data from the last European *Bacteroides* antibiotic susceptibility study [2]. In general, the resistance data for Romania followed the European trends with high ampicillin resistance, moderate or low levels of resistance for cefoxitin, clindamycin, moxifloxacin and amoxicillin/clavulanate and very good data, i.e. very low levels of resistance (<6%) for imipenem, piperacillin/tazobactam, metronidazole and tigecycline. Tetracycline susceptibilities were also measured, and according to world-wide data the resistance was high and has reached 76%. Differencies among the southern and northern European countries were found earlier and also in the latest European *Bacteroides* antibiotic susceptibility study for cefoxitin, moxifloxacin and clindamycin, where cefoxitin and moxifloxacin resistances were higher in northern countries, but in these countries lower clindamycin levels were observed [2,8]. The resistance values obtained in this study for cefoxitin, moxifloxacin and clindamycin lay between the corresponding data values for Eastern European countries. A recent study from Bulgaria reported similarly low resistance values for cefoxitin (1%), clindamycin (3%) and metronidazole (0%) for *Bacteroides* fragilis group isolates [9]. No significant trends regarding the changes in the antimicrobial resistance levels over time were found. The Romanian *B. fragilis* strains exhibited lower resistance

rates for piperacillin/tazobactam, cefoxitin and clindamycin than non-fragilis *Bacteroides* strains (Table 1).

3.2. Correlation of the resistance data and genetic background

For β-lactam antibiotics (ampicillin, cefoxitin and imipenem), the β-lactamase gene (*cepA*, *cfxA* and *cfiA*) content with MIC distributions are depicted in Fig 1. An increasing tendency to affect the MICs of the strains due to the presence of the β-lactamase genes can be seen in the order of *cepA* < *cfxA* < *cfiA*. Thus the *cepA* gene occurred in the whole ampicillin MIC range, *cfxA* displayed a small dilutions shift towards higher cefoxitin MICs (as was found in earlier reports [10]), and *cfiA* positive strains were separated and had the highest imipenem MIC values. No imipenem resistant *B. fragilis* strains were found, but the 3 *cfiA*-positive strains displayed elevated imipenem MICs (1–2 μg/ml) and no IS element was found in their upstream positions. The prevalence of *cfiA*-positive *B. fragilis* strains (8.3%) corresponds to internationally published values or is somewhat higher. No specific gene could be linked to the observed amoxicillin/clavulanate and piperacillin/tazobactam-resistant cases.

The clindamycin MIC distribution (Fig. 2A) was trimodal with three peaks at 0.064, 1 and 256 μg/ml. The 4 *ermF*-positive strains (7.5%) fell into this latter high resistance category. However, the prevalence of the *ermF* gene (7.5%) was much less than that for the European (24.2%) average, but it was similar to the case of Costa Rica where no *ermF*-positive isolates were found [11] among clindamycin-resistant isolates, despite a 22% resistance rate [12]. Some of the other MLS_B, lincosamide or streptogramin resistance genes (*ermB*, *msrSA*, *linA* and *satG*) were harbored by isolates having MICs in the resistance category (4 μg/ml). We did not find the coincidence *ermG*, *mefA*, *msrSA* genes that would indicate the presence of CTnGERM1 [13]. Four IS*4351* element-positive strains were found, out of which only one harbored an *ermF* gene.

One *nim*-positive strain (2.0%) was found that was not metronidazole-resistant (MIC 0.125 μg/ml). From nucleotide sequencing this *nim* gene turned out to be *nimB* and using plasmid isolation we detected the presence of only two cryptic plasmids (4.2 and 5.6 kb), so this *nimB* gene was probably chromosomal.

Despite the fact that the *tetQ* gene was present in the whole tetracycline MIC range (Fig. 2B), its prevalence was higher in higher tetracyclin MIC categories ($p < 0.001$). Of the 33 *tetQ*-positive strains, only 2 lay in the susceptible range. Here, two *tetX1*-positive and 1 *tetM*-positive strains were detected. This latter coincided with a *tetQ* gene in a strain with 32 μg/ml tetracycline MIC, so its role in the tetracycline resistance of the carrying strain could not be estimated.

Nine *bexA*-positive strains were detected and most of them were harbored by strains that had elevated moxifloxacin MICs (2–4 μg/ml). However, this gene was not found in truly moxifloxacin-resistant strains, hence the usual *gyrA* mutations might lead to these phenotypes [14,15].

4. Conclusions

We were the first to measure the antibiotic resistance levels for relevant antibiotics of *Bacteroides* strains isolated in Romania, and this may help empiric antibiotic therapy options for clinicians in this country in the future. The antibiotic resistance levels found were similar to those found in other East European countries, especially Bulgaria. In addition, as part of an ESGAI study, we detected the prevalence of the corresponding antibiotic resistance genes. An increasing influence of the *cepA*, *cfxA* and *cfiA* genes was observed for the resistance levels of the corresponding antibiotics (ampicillin, cefoxitin and imipenem). The prevalence of other genes

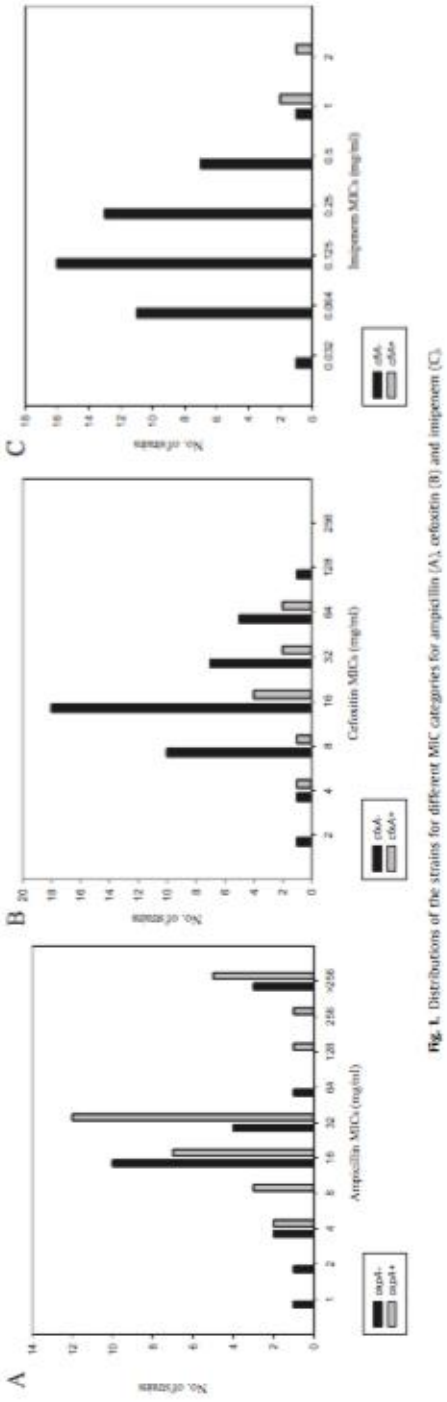


Fig. 1. Distributions of the strains for different MIC categories for ampicillin (A), cefoxitin (B) and imipenem (C).

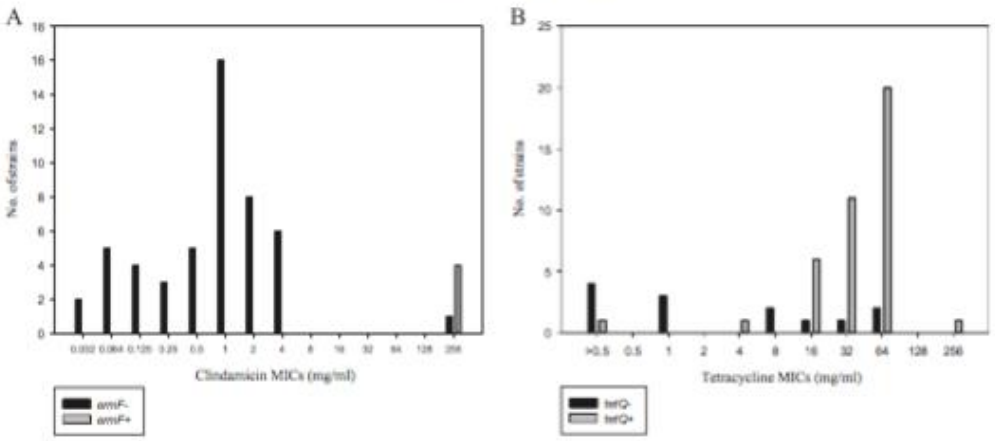


Fig. 2. Distributions of the strains for different MIC categories for clindamycin (A) and tetracycline (B).

displayed a trend similar to East European averages. However, some differencies were also found like a low rate of the *ermF* genes and an absence of the CTnGERM1 element.

Competing interests

None declared.

Acknowledgments

We wish to thank the support from the ESGAI and from the Institute of Clinical Microbiology at Szeged which made this study possible.

References

[1] Snydman DR, Jacobus NV, McDermott LA, Golan Y, Hecht DW, Goldstein EJC, et al. Lessons learned from the anaerobe survey: historical perspective and review of most recent data (2005-2007). Clin Infect Dis 2010;50:S26–33.
[2] Nagy E, Urbán E, Nord CE. Bacteria obotESGoARiA. Antimicrobial susceptibility of Bacteroides fragilis group isolates in Europe 20 years of experience. Clin Microbiol Infect 2011;17:371–9.
[3] Wybo I, Van den Bossche D, Soetens O, Vekens E, Vandoorslaer K, Claeys G, et al. Fourth Belgian multicentre survey of antibiotic susceptibility of anaerobic bacteria. J Antimicrob Chemother 2014;69:155–61.
[4] Karlowsky JA, Walkty AJ, Adam HJ, Baxter MR, Hoban DJ, Zhanel GG. Prevalence of antimicrobial resistance among clinical isolates of Bacteroides fragilis group in Canada in 2010-2011: CANWARD surveillance study. Antimicrobial Agents Chemother 2012;56:1247–52.
[5] Eitel Z, Soki J, Urbán E, Nagy E. The prevalence of antibiotic resistance genes in Bacteroides fragilis group strains isolated in different European countries. Anaerobe 2013;21:43–9.
[6] Sóki J, Eitel Z, Urbán E, Nagy E. Molecular analysis of the carbapenem and metronidazole resistance mechanisms of Bacteroides strains reported in a Europe-wide antibiotic resistance survey. Int J Antimicrob Agents 2013;41:122–5.
[7] Citron DMP IR, Baron EJ. Bacteroides, Porphyromonas, Prevotella, Fusobacterium, and other gram-negative rods. In: Murray PRB EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology. Washington, DC: American Society for Microbiology; 2007. p. 911–32.
[8] Hedberg M, Nord CE, on behalf of the ESGoARiAB. Antimicrobial susceptibility of Bacteroides fragilis group isolates in Europe. Clin Microbiol Infect 2003;9:475–88.
[9] Marina M, Ivanova M, Kantardjiev T. Antimicrobial susceptibility of anaerobic bacteria in Bulgaria. Anaerobe 2009;15:127–32.
[10] Sóki J, Gonzalez SM, Urbán E, Nagy E, Ayala JA. Molecular analysis of the effector mechanisms of cefoxitin resistance among Bacteroides strains. J Antimicrob Chemother 2011;66:2492–500.
[11] Molina J, Barrantes G, Quesada-Gomez C, Rodriguez-Cavallini E. Phenotypic and genotypic characterization of multidrug-resistant *Bacteroides*, *Parabacteroides* spp., and *Pseudofluvonifructor* from a Costa Rican Hospital. Microb Drug Resist 2014 mar 7 [Epub ahead of print] http://dx.doi.org/10.1089/mdr.2013.0180.
[12] Cordero-Laurent ER C, Rodriguez-Cavallini E, del Mar Gamboa-Coronado M, Quesada-Gómez C. Resistance of Bacteroides isolates recovered among clinical samples from a major Costa-Rican hospital between 2000 and 2008 to β-lactams, clindamycin, metronidazole, and chloramphenicol. Rev Esp Quimioter 2012;25:261–5.
[13] Wang Y, Wang G-R, Shelby A, Shoemaker NB, Salyers AA. A newly discovered Bacteroides conjugative transposon, CTnGERM1, containes genes also found in gram-positive bacteria. Appl Environ Microbiol 2003;69:4595–4546–4593.
[14] Bachoual R, Dubreuil L, Soussy C-J, Tankovic J. Roles of gyrA mutations in resistance of clinical isolates and in vitro mutants of Bacteroides fragilis to the new Fluoroquinolone Trovafloxacin. Antimicrobial Agents Chemother 2000;44:1842–5.
[15] Ricci V, Peterson ML, Rotschafer JC, Wexler H, Piddock LJV. Role of Topoisomerase mutations and efflux in Fluoroquinolone resistance of Bacteroides fragilis clinical isolates and laboratory mutants. Antimicrobial Agents Chemother 2004;48:1344–6.

PAPER V

Anaerobe xxx (2014) 1–7

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Development of EUCAST disk diffusion method for susceptibility testing of the *Bacteroides fragilis* group isolates☆

Elisabeth Nagy [a, *], Ulrik Stenz Justesen [b], Zsuzsa Eitel [a], Edit Urbán [a], on behalf of ESCMID Study Group on Anaerobic Infection

[a] Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary
[b] Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Bacteroides fragilis group
Susceptibility testing
Disc diffusion
MIC determination
Breakpoints

ABSTRACT

With the emergence of antibiotic resistance among *Bacteroides fragilis* group isolates the need of susceptibility testing in routine laboratories is increasing. The aims of the present study were to evaluate the disk diffusion method for susceptibility testing in case of different clinical isolates of *Bacteroides* spp by comparing zone diameter results with MICs obtained earlier during an Europe-wide antibiotic susceptibility surveillance, and to propose zone diameter breakpoints, which correlate for the EUCAST MIC breakpoints. We tested 381 clinical isolates of the *B. fragilis* group to amoxicillin/clavulanic acid, cefoxitin, clindamycin, imipenem, metronidazole, moxifloxacin, piperacillin/tazobactam, tigecycline by agar dilution method previously. The inhibition zones of the same antibiotics including meropenem disc were determined by the disc diffusion on Brucella blood agar supplemented with haemin and vitamin K1. Plates were incubated at 37 °C in an anaerobic atmosphere for 24 h. The zone diameters were read at 100% inhibition. In case of discrepant results MICs were determined by gradient test and compared with the inhibition zones on the same plate. We found a good agreement between the inhibition zone diameters and the MICs for imipenem, metronidazole, moxifloxacin and tigecyclin. The inhibition zone diameters of meropenem also separated clearly the isolates, which can be considered wild-type isolates. In case of amoxicillin/clavulanic acid and piperacillin/tazobactam intermediate and susceptible isolates according to the MIC determination, overlap during the zone diameter determination. Isolates with an inhibition zone <23 mm for amoxicillin/clavulanic acid and <25 mm for piperacillin/tazobactam should be retested by a MIC determination method. The 10 μg clindamycin disc clearly separated the resistant and the susceptible population of *B. fragilis* group strains. In the case of cefoxitin only resistant population could be separated with an inhibition zone <17 mm, intermediate and susceptible isolates overlap. In conclusion, we suggest that disk diffusion can be an option for susceptibility testing of *B. fragilis* group isolates for most relevant antibiotics in routine laboratories.

1. Introduction

Antimicrobial susceptibility testing of anaerobic bacteria is important because variable resistance to penicillins, cephalosporins and clindamycin undermines empiric antimicrobial choices. Furthermore, increasing resistance to carbapenems and beta-lactams in combination with beta-lactamase inhibitors are also registered [1,2]. The rate of metronidazole resistance or reduced susceptibility in *Bacteroides* spp. is generally low, around 1%, but some reference laboratories observed an increased number of metronidazole resistant strains (up to 7.5%) among the referred *Bacteroides* isolates at the end of the previous century [3]. This reported trend may be the result of a selection bias as these isolates were referred to the reference laboratory solely to confirm putative resistance to a 5 μg metronidazole disc used in the routine. To identify resistance among anaerobic bacteria including *Bacteroides fragilis* group isolates is important, as anaerobic bacteria are associated with life threatening infections with high morbidity and mortality, especially if inappropriate antimicrobials are administered [4]. Increasing number of cases is reported where multiresistant *Bacteroides* strains are isolated [5–7].

☆ Part of this study was presented during the 23rd ECCMID in Barcelona 2014.

* Corresponding author. Institute of Clinical Microbiology, University of Szeged, Semmelweis u. 6, H-6725 Szeged, Hungary. Tel.: +36 62 541699; fax: +36 62 545712.
E-mail address: nagy.erzsebet@med.u-szeged.hu (E. Nagy).

http://dx.doi.org/10.1016/j.anaerobe.2014.10.008
1075-9964/

Departments of clinical microbiology around Europe are harmonizing their methods for antimicrobial susceptibility testing and classification of resistance. The EUCAST (European Committee on Antimicrobial Susceptibility Testing) disk diffusion susceptibility testing method has been developed for this purpose for aerobic bacteria [8]. Despite of several previous studies [9,10], disk diffusion criteria for antimicrobial susceptibility testing of anaerobic bacteria have not yet been fully determined. To use the disk diffusion method is much less expensive and easier to perform than any other antimicrobial susceptibility testing method based on MIC determination. Data from earlier studies [9,10] and preliminary data from recent studies published during the ECCMID 2013, suggest that it can be further developed for antibiotic resistance determination of rapid growing anaerobic bacteria such as *B. fragilis* group isolates [11].

The objectives of this study were to evaluate disk diffusion for susceptibility testing of *B. fragilis* group strains by comparing disk diffusion results with MICs obtained earlier during a Europe-wide antibiotic susceptibility surveillance study [1] and to propose tentative zone diameter breakpoints which correlate with the EUCAST [8] or where no EUCAST MIC breakpoint is available with CLSI MIC breakpoints [12] published recently.

2. Materials and methods

2.1. Preparation of the inoculum and pre-reduction of the media

The first part of the study was performed in Odense (Denmark) and evaluated the preparation of the inoculum for disc diffusion by using *B. fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 quality control strains recommended by CLSI for antimicrobial susceptibility testing of anaerobic bacteria. Tioglycolate broth and 0.85% saline were compared for preparation of the 1 McFarland inoculum. Parallel measurements of the inhibition zones were carried out comparing zone diameters if culture plates were pre-reduced in an anaerobic atmosphere (10% H_2, 10% CO_2, 80% N_2) or not. The 15-15-15-minute rule of EUCAST was applied. This means that the inoculum suspension is used within 15 min after preparation, the disks are placed on the inoculated plates within 15 min and then the pates are placed in the correct incubation atmosphere (in our case in the anaerobic environment) within another 15 min. For this part of the study piperacillin-tazobactam (30/6 μg/disc), meropenem (10 μg/disc), and metronidazole (5 μg/disc) were used. The results were compared with the Wilcoxon signed rank test and Hodges-Lehmann estimates of median differences with exact 95% confidence intervals were calculated.

2.2. Bacteroides strains and cultivation

Altogether 381 *B. fragilis* group clinical isolates were included this study; they were collected during the last European *Bacteroides* resistance surveillance study in 2007–2008 and stored in a brain–heart infusion (BHI) with 15% glycerin at −80 °C in Szeged, Hungary [1]. *B. fragilis* (272) and other *Bacteroides* species (109) including *B. thetaiotaomicron*, *Bacteroides ovatus*, *Bacteroides vulgatus*, *Parabacteroides (B.) distasonis*, *Bacteroides uniformis*, *Bacteroides eggerthii* and *Bacteroides nordii* were cultured from this collection on Brucella blood agar supplemented with haemin and vitamin K_1 (Becton Dickinson, Heidelberg, Germany) for 48 h in an anaerobic environment (Becton Dickinson GasPak EZ Container System, Heidelberg, Germany). Agar dilution for nine antimicrobial agents (ampicillin, cefoxitin, clindamycin, amoxicillin/clavulanic acid, piperacillin–tazobactam, tigecycline, imipenem–cilastatin, metronidazole and moxifloxacin) has been performed earlier [1]. During the present disk diffusion test the same antibiotics except ampicillin were used and the results were compared with the MIC values determined during the original study. *B. fragilis* ATCC 25285 was used as the control strain for all measurements, using the different antibiotic discs and the corresponding E-test (bioMérieux, Marcy-l'Etoile, France) on the same plate. No inter-laboratory or inter-personal reproducibility evaluation was carried out in this study. All the measurements were done by the same person.

2.3. Susceptibility testing by disk diffusion

All the susceptibility testing by disk diffusion was carried out in Szeged (Hungary). The inoculum from the 24 h primary plates of the isolates was prepared in physiological saline to reach McFarland 1. The 15-15-15-minute rule of EUCAST was used. Nine antibiotics were tested during the disc diffusion measurements on Brucella blood agar supplemented with haemin and vitamin K_1 (Becton Dickinson, Heidelberg, Germany). The antibiotic discs were as follows: amoxicillin/clavulanic acid (20/10 μg/disc), piperacillin/tazobactam (30/6 μg/disc), cefoxitin (30 μg/disc) imipenem/cilastatin (10 μg/disc), meropenem (10 μg/disc), clindamycin (10 μg/disc), tigecycline (15 μg/disc), metronidazole (5 μg/disc), moxifloxacin (5 μg/disc). All discs were obtained from BioRad (Marnes-la-Coquette, France) except metronidazole and clindamycin, which were purchased from Oxoid (Basingstoke, UK). The plates were incubated at 37 °C in an anaerobic atmosphere for 24 h. Zone diameters were read at 100% inhibition. All the measurements were carried out with the naked eye using a caliper. Instead of using regression analysis for the disk diffusion interpretative criteria, in all cases the inhibition zones and the MIC values for the antibiotics were evaluated with the histogram-technique as presented on the EUCAST website [8]. If EUCAST breakpoints were available they were taken into consideration in the evaluation, where only CLSI breakpoints are available (cefoxitin and moxifloxacin) these were taken in consideration. For some strains the disk diffusion results showed very major error (susceptible by disc diffusion and resistant by the previous MIC determination). In all these cases the experiments were repeated using the antibiotic disk and the gradient diffusion MIC determination (E-test strip from bioMérieux, Marcy-l'Etoile, France) on the same plate and same culture conditions. Because of this, it was also possible to evaluate the zone-diameter discrepancies between repeated parallel measurements of 113 organism/antibiotic combinations.

3. Results

Using the *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 control strains the parallel measurements (altogether 24 for both stains) showed that there were no significant differences in the inhibition zone diameters measured for piperacillin/tazobactam, metronidazole and meropenem, when thiogylcolate broth was used for the inoculum preparation and the test was carried out on pre-reduced media, compared with preparation of the inoculum in 0.85% saline and using non-pre-reduced media (data not shown). Accordingly, for the main study 0.85% saline solution was used for the preparation of the inoculum and the plates were not pre-reduced.

A total of 381 *B. fragilis* group clinical isolates were tested. The distribution of the inhibition zone diameters and MICs for the eight antibiotics where MIC data were available is illustrated in the histograms (Fig. 1) as used by EUCAST for demonstrating the relationship between MICs and zone diameters. In the case of imipenem (Fig. 1A) relatively few isolates were resistant (no. 4) or intermediate susceptible (no. 2). For each MIC value the inhibition zones varied from 0 to 13 mm, with 90% of the values within 8 mm. The resistant isolates were clearly separated from the susceptible

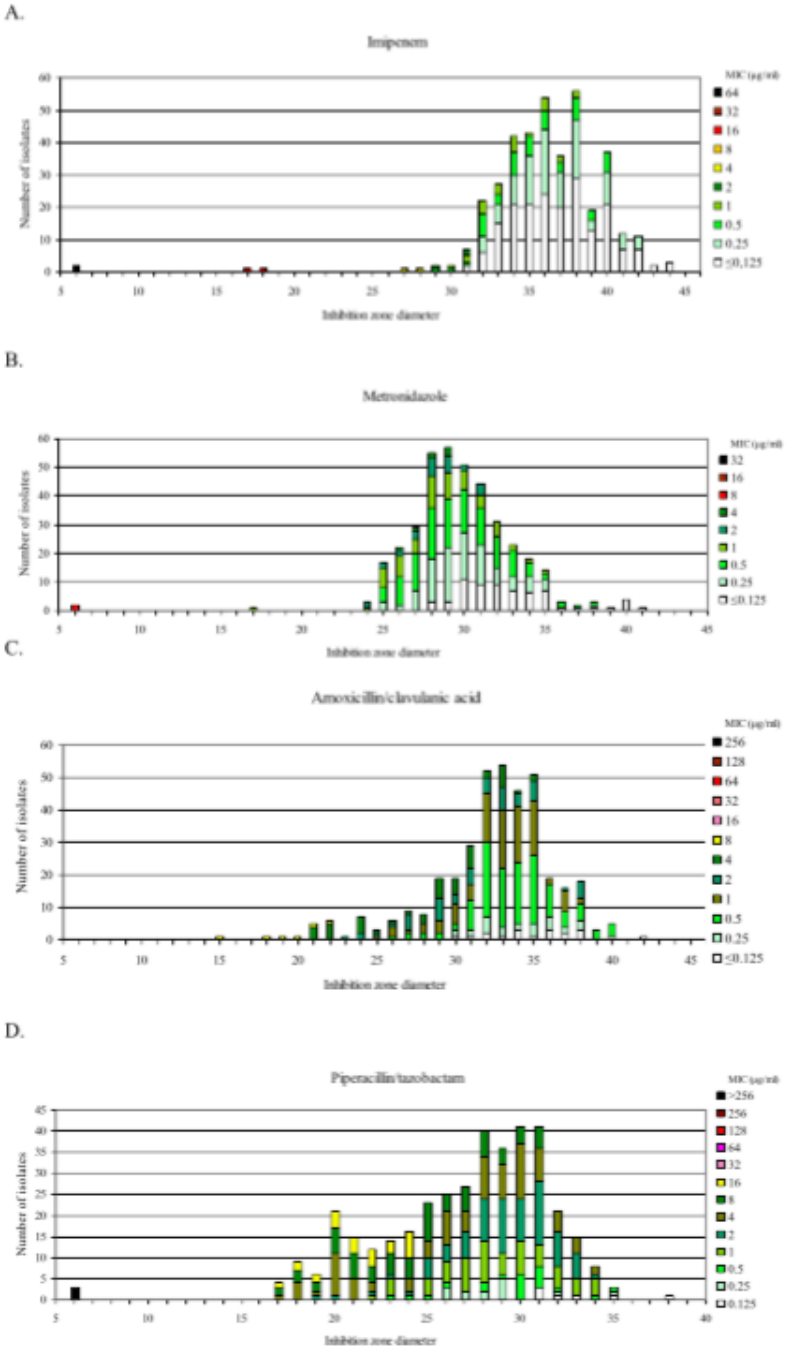
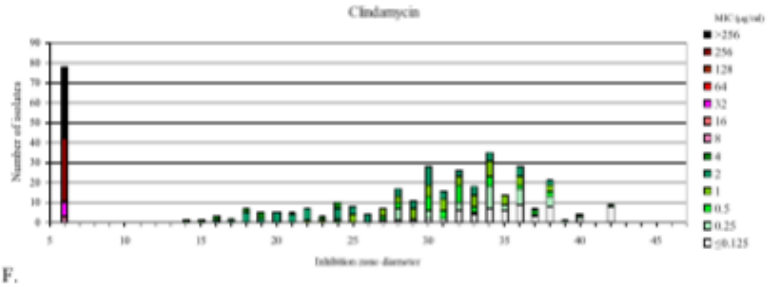
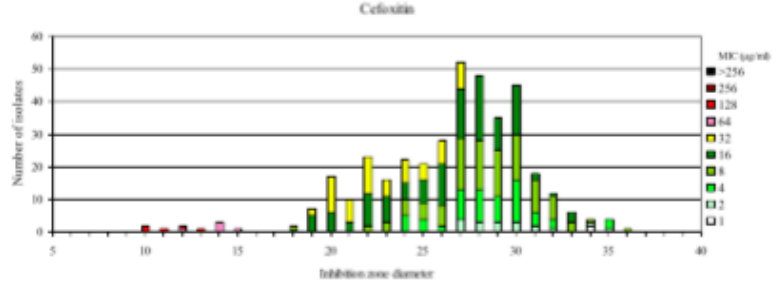


Fig. 1. Distribution of inhibition zone diameters and MICs for 381 *B. fragilis* group isolates A: imipenem (10 μg disk), B: metronidazole (5 μg disk), C: amoxicillin/clavulanic acid (20/10 μg disk), D: piperacillin/tazobactam (30/6 μg disk), E: clindamycin (10 μg disk), F: cefoxitin (30 μg disk), G: moxifloxacin (5 μg disk), H: tigecycline (15 μg disk). Each isolate is shown in the zone diameter histogram in a colour representing its MIC value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

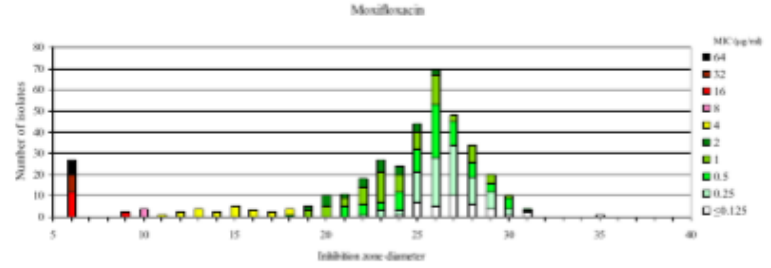
E.



F.



G.



H.

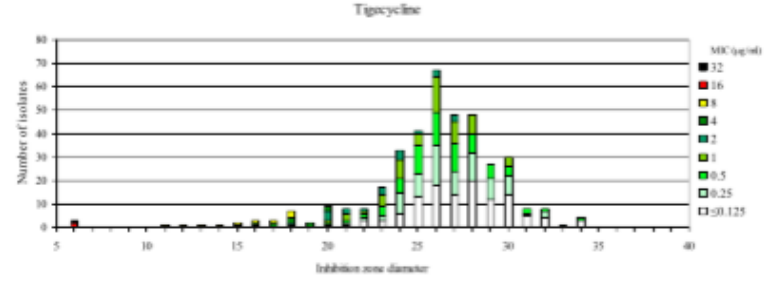


Fig. 1. (continued).

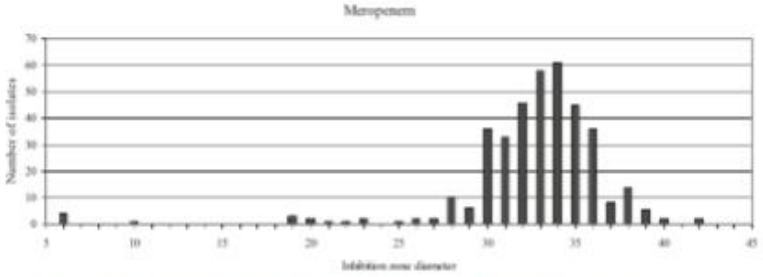


Fig. 2. Distribution of inhibition zone diameters of meropenem (10 μg) disk for 381 *B. fragilis* group isolates.

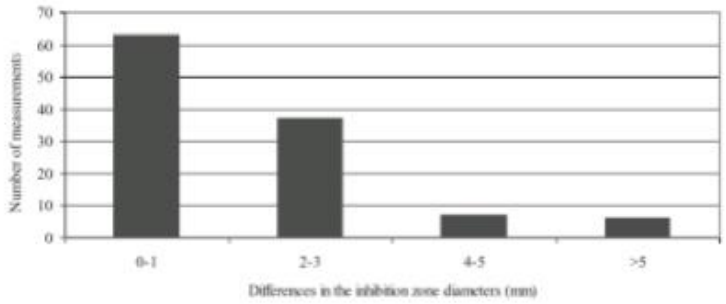


Fig. 3. Zone diameter differences between repeated measurements (altogether 113).

strains: inhibition zone diameter for the resistant strains was ≤20 mm and for the susceptible strains ≥29 mm, respectively. The same was true for metronidazole (Fig. 1B) where only 2 isolates were found, which were resistant according to the EUCAST breakpoints, with an MIC 8 μg/ml. All but one of the susceptible isolates had an inhibition zone ≥24 mm. The inhibition zones for the different MICs varied between 0 and 14 mm and 90% of the values were within 6 mm.

According to the MIC data no amoxicillin/clavulanic acid resistant isolates were among the strains tested (Fig. 1C). A very large distribution of the inhibition zones of the strains with the same MICs was seen (8–14 mm), however intermediate resistant strains (MIC 8 μg/ml) had an inhibition zone ≤22 mm with some overlap with susceptible isolates.

In the case of piperacillin/tazobactam (Fig 1. D), three resistant strains were found clearly separated from the intermediate and susceptibly population by an inhibition zone ≤16 mm, however intermediate and susceptible isolates overlapped by the disc diffusion method. According to these data, isolates with an inhibition zone ≤24 mm and >16 mm should be tested by the *E*-test to determine MICs and differentiate fully susceptible from intermediate susceptible isolates. The distribution of inhibition zone diameters for each MIC values varied between 0 and 17 mm.

For testing clindamycin susceptibility of the strains (Fig. 1E) by disc diffusion instead of the 2 μg/disc the 10 μg/disc was used. This clearly separated the resistant population with an inhibition zone ≤13 mm. The results showed that the inhibition zone diameters for the clindamycin susceptible strains stretched out between 14 and 42 mm, however the very susceptible (MIC ≤ 0.125 μg/ml) isolates had a larger inhibition zone diameter in average than those having higher MICs. The distribution of inhibition zone diameters for each MIC values varied between 0 and 23 mm.

In the case of cefoxitin only CLSI breakpoints are available (Fig. 1F). According to those the susceptible isolates had inhibition zone diameters between 18 mm and 36 mm. The isolates with intermediate MICs had inhibition zone between 18 mm and 27 mm,

Table 1
EUCAST MIC breakpoints for the antibiotics tested and the suggested susceptibility zone diameter breakpoints.

Antimicrobial agents	EUCAST clinical MIC breakpoints (mg/L)		Zone diameter breakpoints (mm)
	S≤	R>	S≥
Imipenem	2	8	29
Metronidazole	4	4	24
Amoxicillin/clavulanic acid	4	8	15
Piperacillin/tazobactam	8	16	25
Clindamycin	4	4	25
Cefoxitin	16[a]	32[a]	?
Moxifloxacin	2[a]	4[a]	19

?: means that no zone diameter sensitivity breakpoint decision was possible.
[a] Only CLSI breakpoints are available.

but this range overlapped with the fully susceptible isolates. However, the resistant population was separated from the susceptible isolates with an inhibition zone ≤15 mm. The zone diameters varied for the different MICs between 0 and 15 mm.

For moxifloxacin only CLSI breakpoints are available (Fig. 1G). The disk diffusion test clearly separated the susceptible isolates with an inhibition zone ≥19 mm. Only few isolates were found in the intermediate range with an inhibition zone between 11 mm and 18 mm. All the resistant isolates had a zone diameter ≤10 mm. The zone diameters varied for the different MICs between 0 and 11 mm with 95% within 6 mm.

For tigecycline (Fig. 1H) no MIC breakpoints are available in the EUCAST or CLSI documents, accordingly only comparison of the MICs and the zone diameters was possible. Among the strains tested only three were found which could be considered fully resistant and had no inhibition zone at all with MICs ≥32 μg/ml. All the strains which had MICs ≤4 μg/ml could be separated with a zone diameter ≥20 mm. For each MIC value the inhibition zones varied from 0 to 11 mm, with 90% of the values within 6 mm.

For meropenem we did not have MIC data from the previous European surveillance study. Fig 2 only shows the distribution of the zone diameters. Most of the strains had a zone diameter ≥28 mm, which separated the few isolates, which can be considered intermediate susceptible or resistant to carbapenems. If we compared the distribution of the zone diameters of imipenem and meropenem more isolates not belonging to the wild type strains could be detected by the meropenem disc. This is in agreement with some other studies where meropenem could better distinguish between carbapenem susceptible and intermediate susceptible isolates [13].

In those cases where discrepant results (very major errors) were found for the disc diffusion zone diameter data and the agar dilution MIC determination data carried out more than 6 years ago, the measurements were repeated by *E*-test and disc diffusion on the same plate. Data of these repeated measurements were taken in consideration during the final data processing. The evaluation of results of the repeated disk diffusion measurements gave also an opportunity to check the reproducibility of the measurements of the inhibition zones. Out of the 113 measurements (different isolates and different antibiotic discs), 88.5% of the repeated measurements were within 0–3 mm (Fig. 3). The standard deviations of the zone diameters for the *B. fragilis* ATCC 25285 based on 15 parallel measurements were between 0.5 and 2.2 for the different antibiotics (data not shown).

According to our experiments the tentative zone diameter susceptibility breakpoints for the different antibiotics compared with the MIC breakpoints set by the EUCAST or CLSI are presented in Table 1.

4. Discussion

Antibiotic susceptibility testing of anaerobic bacteria is becoming more and more important for the clinical microbiological laboratories, as the susceptibility of anaerobic bacteria, especially *B. fragilis* group strains have become increasingly unpredictable during the past decades. Resistance to the most active antibiotics such as carbapenems piperacillin/tazobactam, amoxicillin/clavulanic acid and metronidazole has been reported during nation-wide or European studies [1,2,14,15]. Additionally there are clear differences in the resistance according to the geographical areas [1] and even differences can be found in resistance patterns in different hospitals in a single city, which may be due to the variability in selecting antibiotics for empirical treatment [2]. Several papers reported about cases caused by broadly multidrug-resistant *B. fragilis* isolates [6,7,16,17], as well as number of reports describes the development of resistance in vitro or in vivo in the presence of antibiotics [18,19]. These events emphasize the need for antimicrobial susceptibility testing of anaerobes not only during periodical surveillances in different geographical areas, but also during the everyday routine laboratory testing. Among the methods which are standardized today for antibiotic susceptibility testing of anaerobic bacteria the gradient test (*E*-test) is used in most laboratories which carry out resistance determination for selected anaerobic isolates for few antibiotics and in special cases [20,21], but for many laboratories in low income countries it is too expensive to be used on the regular basis. Other methods such as the agar dilution method are not for routine use. With the broth microdilution MIC determination method, using small amounts of antibiotic containing broth, it is difficult to achieve a proper anaerobe environment during inoculation. The final result may be influenced by this, especially in slow growing anaerobes. The spiral gradient endpoint system is not frequently used in European routine laboratories. For rapid growing anaerobes (*Bacteroides* spp. or most of the clostridia) disc diffusion method could be a solution. Earlier several groups used this method for evaluation the activities of anti-anaerobic drugs, however standardization of culture circumstances (media, inoculum and incubation) were not fully solved [22–24]. EUCAST already published its own breakpoints for some of the anti-anaerobic drugs, which can be applied for agar dilution MIC determination [8]. The first study to set up zone diameter breakpoints for *Clostridium difficile* has been published recently [25] with the conclusion that among the testing conditions used in the study, the disc diffusion method is a reliable option for antimicrobial susceptibility testing of *C. difficile*. There are already publications about *C. difficile* infections caused by isolates with reduced susceptibility to metronidazole or vancomycin as well as ~~about~~ the increasing number of strains detected to be resistant to moxifloxacin or rifaximin. It will probably be desirable in the future to monitor the resistance patterns of *C. difficile* isolates in routine laboratories by a simple method [25]. Extensive study was carried out to find the proper media for the disc diffusion testing of rapid growing *Bacteroides* isolates as well [26,27] and the first evaluation was presented during the ECCMID 2013 [11]. In our study we used standardized inoculum preparation, the supplemented Brucella blood agar obtained from the same supplier through out of the study and a fully controlled anaerobic incubation for exactly 24 h. A large selection of *B. fragilis* and other *Bacteroides* spp were tested against 9 antibiotics with differences in resistance rates against these antibiotics. The only draw back of the study was that MIC data were collected more that 6 years ago for the strains, which were stored in −80 °C during this time. This may result changes in the resistance levels of some isolates. However, discrepant results, in most cases very major errors (susceptible by disc diffusion and resistant by the previous MIC determination) were cleared up by repeated measurements on the same plate with the *E*-test and the disc diffusion. At the final evaluation no very major errors were found. Major errors (resistant by disc diffusion and susceptible/intermediate susceptible by the MIC determination) were found in one isolate for metronidazole and one isolate for cefoxitine. Reproducibility of the disc diffusion measurements for the control strains were carried out in two different locations. In Odense during testing the composition of the broth for the inoculum and the effect of the pre-reduction of the plates no significant differences were observed in the zone diameters for meropenem, metronidazole and piperacillin/tazobactam for *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741. However, it should be emphasized that following the EUCAST 15-15-15 min rule strictly, as well as usage of the proper anaerobe environment for the culture is a prerequisite to obtain comparable results. Pre-reduction of the broth for inoculum preparation, as well as the use of the pre-

reduced media may be a prerequisite for testing other, more oxygen susceptible anaerobic bacteria. In Szeged the standard deviation of zone diameters during the parallel measurements of the 9 antibiotics for the *B. fragilis* ATCC 25285 were between 0.5 and 2.2 mm, independent of the time points of the measurement, or the lots of the media.

With one exception (cefoxitin) we could suggest tentative zone diameter breakpoints for susceptible *B. fragilis* group strains. We found a good agreement between the inhibition zone diameters and the MICs for imipenem, metronidazole, moxifloxacin and tigecyclin similar to the previous study testing 104 *B. fragilis* group isolates [11]. The inhibition zone diameters of meropenem also separated clearly the isolates, which can be considered wild-type isolates. In case of amoxicillin/clavulanic acid and piperacillin/tazobactam intermediate and susceptible isolates according to the MIC determination overlap during the zone diameter determination. Isolates with an inhibition zone <23 mm for amoxicillin/clavulanic acid and <25 mm for piperacillin/tazobactam should be retested by a MIC determination method to differentiate susceptible and intermediate susceptible strains. As a difference from previous studies [11,22], the 10 μg clindamycin disc was used during this study instead of the 2 μg. A clear separation between the resistant and the susceptible population of the *B. fragilis* group strains was observed with the higher content clindamycin disc. However, we have not evaluated its applicability for the detection of the inducible clindamycin resistance. In the case of cefoxitin only resistant population could be separated with an inhibition zone <17 mm, intermediate and susceptible isolates overlap using the disc diffusion. No zone diameter breakpoint can be suggested for susceptibility to cefoxitin according to our data. In conclusion, we suggest that disk diffusion method may be an option for susceptibility testing of *B. fragilis* group isolates for most relevant antibiotics in routine laboratories. Further studies are needed to evaluate the disc diffusion method for other anaerobic bacteria.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgements

This study was supported by a research grant from ESCMID for the ESCMID Study Group for Anaerobic Infections (ESGAI).

References

[1] Nagy E, Urbán E, Nord C-E. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. Clin Microbiol Infect 2011;17:371–9.
[2] Snydman DR, Jacobus NV, McDermott LA, Golan Y, Hecht DW, Goldstein EJ, et al. Lessons learned from the anaerobe survey : historical perspective and review of the most recent data (2005–2007). Clin Infect Dis 2010;50(Suppl. 1):S26–33.
[3] Brazier JS, Stubbs SLJ, Duerden BI. Metronidazole resistance among clinical isolates belonging to the *Bacteroides fragilis* group : time to be concerned. J Antimicrob Chemother 1999;44:580–1.
[4] Nguyen MH, Yu VL, Morris AJ, MyDermott L, Wagener MW, Harrell L, et al. Antimicrobial resistance and clinical outcome of *Bacteroides* bacteraemia: findings of a multicenter prospective observational trial. Clin Infect Dis 2000;30:870–6.
[5] Hartmeyer GN, Söki J, Nagy E, Justesen US. Multidrug-resistant *Bacteroides fragilis* group on the rise in Europe? J Med Microbiol 2012;61:1784–8.
[6] Kalapila A, Pergam S, Pottinger P, Butler-Wu S, Whimbley E. Multidrug-resistant *Bacteroides fragilis* – Seattle, Washington 2013. MMWR 2013;62(34):694–6.
[7] Wareham DW, Wilks M, Ahmed D, Brazier JS, Millar M. Anaerobic sepsis due to multidrug-resistant *Bacteroides fragilis*; microbiological cure and clinical response with linezolid therapy. Clin Infect Dis 2005;40:e67–68.
[8] European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoints. 2014. http://www.eucast.org/clinical_breakpoints.
[9] Horn R, Bourgault A-M, Lamothe F. Disk diffusion susceptibility testing of the *Bacteroides fragilis* group. Antimicrob Agents Chemother 1987;31:1596–9.
[10] Barry AL, Fichs PC, Gerlach EH, Allen SD, Acar JF, Aldridge KE, et al. Multilaboratory evaluation of an agar diffusion disk susceptibility test for rapid growing anaerobic bacteria. Rev Infect Dis 1990;12(Suppl. 2):S210–7.
[11] Lau H, Thomsen MD, Hansen F, Citron DM, Kahlmeter G, Justesen US. Disk diffusion antimicrobial susceptibility testing of the *Bacteroides fragilis* group using EUCAST clinical MIC breakpoints ECCMID. 2013. Poster 1577.
[12] Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved Standard. 8th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2012. M11-A8.
[13] Topcak NU, Uzunkaya OD, Söki J, Soyletir G. Susceptibility profiles and resistance genes for carbapenems (*cfiA*) and metronidazole (*nim*) among *Bacteroides* species in a Turkish University Hospital. Anaerobe 2012;18:169–71.
[14] Behra-Miellet J, Calvet L, Mory F, Muller C, Chomarat M, Bézian MC, et al. Antibiotic resistance among anaerobic Gram-negative bacilli; lesson from a French multicentric survey. Anaerobe 2003;9:105–11.
[15] Wybo I, Van den Bossche D, Soetens O, Vekens E, Vandoorslaer K, Claeys G, et al. Fourth Belgian multicentre survey if antibiotic susceptibility of anaerobic bacteria. J Antimicrob Chemother 2014;69:155–61.
[16] Rotimi VO, Khoursheed M, Brazier JS, Jamal WY. *Bacteroides* species highly resistant to metronidazole: an emerging clinical problem? Clin Microbiol Infect 1999;5:166–8.
[17] Katsandri A, Papaparaskevas J, Pantazatou A, Petrikkos GL, Thomopoulos G, Houhoula DP, et al. Two cases od infections due to multidrug-resistant *Bacteroides fragilis* group strains. J Clin Microbiol 2006;44:3465–7.
[18] Turner P, Edwards R, Weston V, Gazi A, Ispahani P, Greenwood D. Simultaneous resistant to metronidazole, co-amoxiclav, and imipenem in clinical isolate of *Bacteroides fragilis*. Lancet 1995;345:1275–6.
[19] Löfmark S, Fang H, Hedberg M, Edlund C. Inducible metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. Antimicrob Agents Chemother 2005;49:1253–6.
[20] Hecht DW. Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. Anaerobe 2006;12:115–21.
[21] Brook I, Wexler HM, Goldstein EJ. Antianaerobic antimicrobials: spectrum and susceptibility testing. Clin Microbiol Rev 2013;26:526–46.
[22] Callihan DR, Nolte FS. Disc diffusion method to screen for high-level resistance to clidamycin and erythromycin in the *Bacteroides fragilis* group. Diagn Microbiol Infect Dis 1985;3:131–7.
[23] Ottmaz M, Benn R. Sensitivity of *Bacteroides fragilis* to seven antimirobial agents. Pathology 1981;13:697–703.
[24] Luzzi I, Pantosti A, Gianfrilli P, Panichi G. Susceptibility of *Bacteroides fragilis* to cefotaxim and piperacillin: tentative interpretatative standards for disc-diffusion test. Eur J Clin Microbiol 1984;3:37–9.
[25] Enkstrup LT, Danielsen TKL, Hall V, Olsen KE, Kristensen B, Kahlmeter G, et al. Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disc diffusion correlates. Clin Microbiol Infect 2012;18:E266–72.
[26] Justesen US, Danielsen TK, Matuschek E, Kahlmeter G. The Brucella blood agar for disk diffusion antimicrobial susceptibility testing of anaerobic bacteria? Clin Microbiol Infect 2011;17(Suppl. 4):S748.
[27] Justesen US, Matuschek E, Kahlmeter G. The EUCAST Mueller-Hinton fastidious agar for antimicrobial susceptibility testing of anaerobic bacteria? Clin Microbiol Infect 2011;17(Suppl. 4):S174.