Epidemiological investigation of toxin producing *Clostridium difficile* and antibiotic resistant *Bacteroides* strains with the use of traditional and molecular biological methods

PhD Thesis

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Abbreviations

5-NI	5-nitro imidazole
AAC	antibiotic-associated colitis
AAD	antibiotic-associated diarrhoea
AP-PCR	arbitrarily primed polymerase chain reaction
ARU	Anaerobe Reference Unit
BHI	brain heart infusion
bp; kb	basepairs; kilobase pairs
CCFA	cycloserine-cefoxitin fructose agar
CDAD	*Clostridium difficile*-associated disease
*cfi*A	gene for cefoxitin-imipenem resistance
DNA	desoxyribonucleic acid
EDTA	ethylendiamine tetraacetic acid
ELFA	enzyme linked fluorescent assay
FAA	Fastidious Anaerobic Agar
IS	insertion sequence
kDa	kilodalton
LPS	lipopolysaccharide
MIC	minimal inhibitory concentration
Mw	molecular weight
NCTC	National Collection of Type Cultures
nim	nitro imidazole resistance gene
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pI	isoelectric point
PMC	pseudomembranous colitis
PMS	pyrolysis mass spectrometry
RAPD	random amplified polymorphic DNA
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
RFV	relative fluorescence value
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS-PAGE	sodium dodecylsulphat polyacrylamide gel electrophoresis
SPR	solid-phase receptacle

1. INTRODUCTION

Anaerobic bacteria have been of microbiological and clinical interest since Louis Pasteur's use of the word "anaérobies" to describe his isolation and description of "septic vibrio" (*Clostridium septicum*). More than 100 years has passed since then, accompanied by ever expanding knowledge about anaerobic bacteria, their pathogenicity and the wide spectrum of anaerobic infections caused by both invasion and intoxication. Anaerobic bacteria are widely distributed in nature in oxygen-free habitats. Many members of the indigenous human flora are anaerobic bacteria, including spirochetes and Gram-positive and Gram-negative cocci and rods. Anaerobic bacteria are important pathogens in a wide variety of infections throughout the human body. They generally originate from the patient's indigenous flora and they may be involved in essentially any type of bacterial infection in humans.

They play an important role in the most commonly encountered categories of infection: osteomyelitis and skin and soft tissue, pleuropulmonary, intraabdominal and female genital tract infections. Most often they are part of a mixed flora, but some infections involve only anaerobes. The severity of anaerobic or mixed anaerobic infections is quite variable, ranging from very low grade (even inapparent) infection to rapidly progressive infection with a significant mortality rate. Infections involving anaerobic bacteria are characterized in particular by a tendency towards suppuration or abscess formation and the production of tissue necrosis. Proliferation of anaerobic bacteria in tissue depends on the absense of oxygen. Oxygen is excluded from the tissue when the local blood supply is impaired by trauma, obstruction or surgical manipulation. Anaerobes multiply well in dead tissue. The multiplication of aerobic or facultative organisms in association with anaerobes in infected tissue also diminishes the oxygen concentration and develops a habitat that supports the growth of anaerobic bacteria (1).

Clostridium difficile is recognized as the major infectious agent responsible for nosocomial diarrhoea and potentially lethal pseudomembranous colitis (PMC) following antimicrobial therapy. Members of the *Bacteroides fragilis* group of anaerobes are the most frequently encountered anaerobic pathogens in clinical infections, and play the most important role of poly- or sometimes monobacterial infections. However, antibiotic resistance is increasingly common among the members of this group. Knowledge of the status and the mechanisms of resistance is critical for both the selection of antimicrobial therapy and the design of new antimocrobial agents. The purpose of this thesis was to investigate the pathogenic role of *C. difficile* in nosocomial infections and to study the mechanisms for and the prevalence of β–lactam and metronidazole resistance in strains belonging in the *B. fragilis* spp.

1.1. Microbiology and epidemiology of *Clostridium difficile* infection

1.1.1. Historical perspective

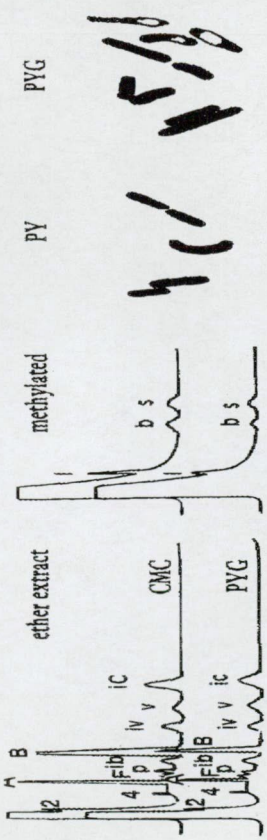
C. difficile made its first appearance in the literature when Hall and O'Toole (1935) described *Bacillus difficilis* as part of the bacterial flora of the meconium and faeces of infants (2). Snyder performed a follow-up study, and found *B. difficilis* in approximately 10% of infant faecal samples (3). These early workers demonstrated that some strains of this anaerobic organism could produce a lethal toxic effect when broth culture filtrates were injected either subcutaneously, intraperitoneally or intravenously into laboratory animals. Although they postulated that *B. difficilis* toxins liberated in the infant gut could play a role in diarrhoea, the presence of occult blood in the stools, and convulsions of the newborn, it was to be almost four decades before the pathogenic potential of *C. difficile* was to become apparent. It was 1969 when the first real clue to the pathogenic potential of this organism to mammals in the absence of competing colonic microbiota was provided (4). In experiments on germ-free rats, it was noted that monocontamination with *C. difficile* often led to the development of transient diarrhoea, which ocassionally caused death. As the organism was an obligately anaerobic Gram-positive spore-bearing bacillus, it was subsequently classified as belonging in the genus *Clostridium,* and reports of *C. difficile* in the literature were mainly limited to chance findings in studies directed at other areas. Three independent studies, concluded in 1974, provided the platform from which *C. difficile* was shown to be an important cause of disease in man. In the USA, Green described a cytotoxin that was present in the stools of guinea-pigs which developed gut disease after receiving penicillin (5). Tedesco *et al.*, also in the USA, found a significant association between patients receiving clindamycin and the development of PMC (6). At this stage, neither group knew the organism responsible for their independent observations. Meanwhile, in the UK, Hafiz completed his PhD thesis on *C. difficile,* but was unaware that the organism he was studying was responsible for the toxic effects noted by others (7). These independent publications were a catalyst to the studies by Bartlett *et al.*, who described a clindamycin-induced colitis caused in hamsters by an unidentified *Clostridium* sp., which was eventually confirmed to be *C. difficile* (8). Larson *et al.* demonstrated that a cytotoxin could also be detected in the stools of 5 out of 6 patients with histologically proven PMC (9). Thus, it was not until the late 1970s that cumulative researches pointed the finger of suspicion at *C. difficile* as an important cause of nosocomial morbidity and mortality. At this time, elucidation of this "new" gut pathogen presented a

challenge to diagnostic clinical microbiology laboratories, which, as time progressed, were met with increasing requests to investigate stool specimens for evidence of *C. difficile* (10).

1.1.2. Microbiology

C. difficile is a spore-forming Gram-positive obligate anaerobic rod, 3-5 μm in length. Sporulation is most noticeable on agar cultures that have reached a stationary or decline phase after >72 h of incubation. The colonial morphology can be quite variable; typically, at 24-48 h on blood-based media, colonies are 3-5 mm in diameter with an irregular, lobal or rhizoidal edge, grey, opaque and non-haemolytic, although some strains have a greenish apperance due to alpha-type haemolysis on blood agar. After 48-72 h of incubation, colonies may develop a light grey or whitish centre, a factor associated with sporulation. This centre is sometimes raised, with an inner concentric margin, giving almost a "fried–egg" appearance. Selective agar media, cycloserine-cefoxitin fructose agar (CCFA) have been used in attempt at the selective isolation of *C. difficile* from faecal samples (11).

Table 1: Biochemical characterization of *C. difficile*



Lecithinase	-
Lipase	-
Gelatine hydrolysis	+
Casein hydrolysis	-
Indole	-
Aesculin pH	-
Aesculin hydrolysis	+
Glucose fermentation	+
Maltose	-
Lactose	-
Saccharose	-
Mannite	+/-
Starch hydrolysis	-
Haemolysis	-
Oxidase, catalase	-
UV fluorescence under 365 nm	Yellow-green or chartreuse
Odour	"Elephant or horse" manure

1.1.3. Epidemiology

C. difficile is normally a harmless environmental organism. As is the case with some other bacterial species, it is man's intervention which has facilitated the conditions whereby it may cause significant human morbidity and mortality. It is the compromise of resistance to colonization afforded by the normal gut flora, usually by antimicrobial agents, that facilitates infection by *C. difficile*.

Asymptomatic colonization is very common in neonates and it has been difficult to attribute any disease manifestation to this group. However, *C. difficile* as a cause of enteric disease cannot be ignored completely, especially in older children with severe underlying disease. Prevalence studies in infants have shown that healthy newborns frequently become colonized with *C. difficile* during the first 2 weeks of life. Acquisition frequencies of 15-63% or 0-6% have been reported (12). Such marked differences probably reflect different degrees of nosocomial environmental exposure in different nurseries, rather than differences in diet or rates of maternal colonization. Neonatal colonization is almost always asymptomatic, despite the presence of strains that are highly toxigenic *in vitro* and similar to those isolated from patients with PMC. It remains unclear why neonates, who are in the process of producing a complex gut bacterial ecosystem, remain unaffected, even though *C. difficile* and its toxins are frequently present in their faeces. The absence of such symptoms could be due to the immature intestinal flora and lack of development of the toxin receptors in the intestine. After the first 2 weeks of life, colonization rates remain constant until about 1-2 years of age. Reported carrier rates in children >2 years of age are <4% (13).

Reported carrier rates in healthy adults have varied from 0.3% in Europe to 15% in Japan. These differences probably reflect differences in the sensitivity of the culturing methods and in selecting subjects who have not previously received antibiotics. In healthy volunteers given antibiotics, asymptomatic carriage has increased to 46% (14).

The incidence of *C. difficile* infections seems relatively low in hospitals, but the carriage and acquisition rates of *C. difficile* are higher. Many teams have focused on the transmission rates of *C. difficile* through prospective studies in which patients were systematically screened for *C. difficile* shortly after their admission into hospital, and then once or twice weekly thereafter (15-17). In these studies, carriage rates at admission range from 5.9 up to 11%. This is a higher rate than the 3% reported in healthy adults, and may reflect the previous administration of antibiotics or the nosocomial acquisition of *C. difficile* during previous hospitalizations. The rate of *C. difficile* acquisition varies with the patient population studied, the use of antibiotics and the presence of an outbreak in the ward studied.

In the absence of an outbreak, the acquisition rate has been estimated at 4-21%, but this acquisition remains asymptomatic in more than 63% of the cases (18-20). Nevertheless, during outbreaks, higher acquisition rates have been observed, such as 32% in the study performed by Delmée in a haematology ward (21).

1.1.4. Pathogenesis and clinical manifestations of *C. difficile* diarrhoea and colitis

C. difficile strains usually produce at least two toxins, called A and B; they are unusually large proteins and share a number of similar features. They are thought to be primarily responsible for the virulence of the bacterium and the major contributors to the pathogenesis of gastrointestinal disease. Toxins A and B have been purified and their molecular and biological properties have been extremely well characterized. (Table 2).

Table 2: Molecular and biological properties of *C. difficile* toxins (22)

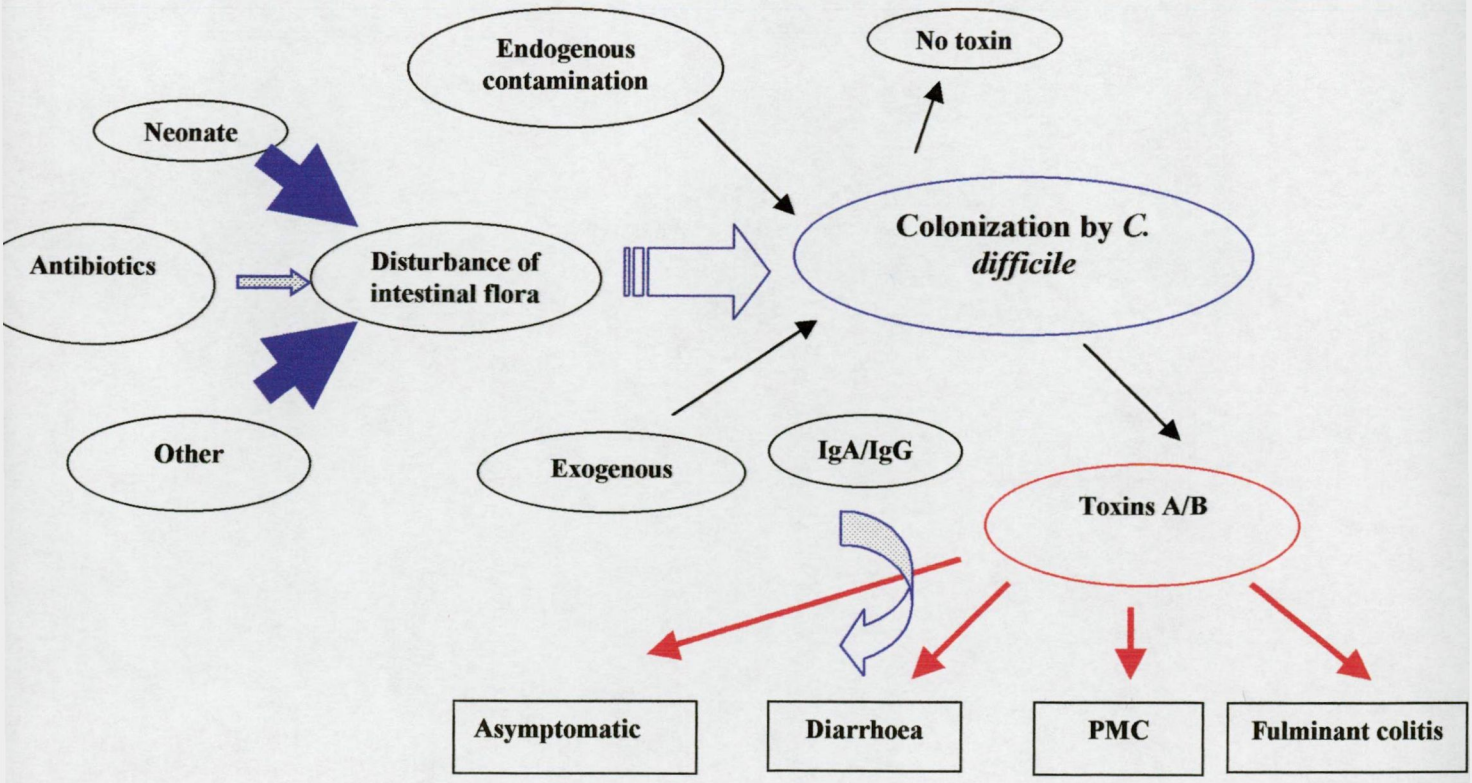
Property	Toxin A	Toxin B
Mw	308 000	270 000
pI	5.6	4.2
Cytotoxicity	10 ng	1 pg
Lethal dose	50 ng	50 ng
Enterotoxicity	1 µg	-
Haemagglutination	+	-
Glucosyltransferase	+	+

Toxin B is active against every cell line which has been tested. Toxin A, though far less cytotoxic on most cell lines as compared with toxin B, is a potent cytotoxin for epithelial cells of intestinal origin such as HT-29 cells. These cells have a high density of carbohydrate receptors for toxin A. Both toxins are glucosyltransferases that covalently modify the Rho proteins. Toxin A is an extremely potent enterotoxin, microgram amounts causing fluid secretion in animal loops, whereas toxin B does not cause damage or a fluid response when injected alone in intestinal loops. This is probably due to an inability to bind to a receptor on the intestinal brush border membrane cells under normal physiological conditions. Toxin A binds to specific carbohydrate receptors on the surface of intestinal cells and initiates damage to the intestines. Toxin A is more resistant than toxin B to proteases such as trypsin and chymotrypsin and to extremes of pH. Both toxins can be protected by reducing agents and are

inactivated by oxidizing agents. Toxin B has a high negative surface charge at neutral pH, whereas toxin A has very little surface charge (23).

The bacterium is known as the aetiological agent of PMC, with 90-100% of the cases, involving antibiotic-associated diarrhoea (AAD) and colitis (AAC) (24,25). Disease may be associated with a spectrum of severity, ranging from mild diarrhoea (most common), through moderately severe disease with watery diarrhoea, abdominal pain and a systemic upset, life-threatening and sometimes fatal PMC. This may be accompanied by toxic megacolon (rare), an electrolyte imbalance and occasional bowel perforation (26-30). One of the most remarkable characteristics of such diseases is that, in almost all cases, they occur following antibiotic therapy. The pathogenesis of *C. difficile*-associated disease (CDAD) is very particular and allows a better understanding of the circumstances in which the clinician should make the diagnosis (23,24,28,31). (Fig. 1)

Fig. 1: Pathogenesis of *C. difficile*-associated diarrhoea (31)



Toxin-producing strains are the most frequently identified cause of nosocomial diarrhoea. Nosocomial acquisition in asymptomatic and symptomatic patients, together with the contamination of the hospital environment and the hands of healthcare workers, has been clearly demonstrated (14,15,19). This bacterium also has a major clinical impact on immunosuppressed hosts, patients in Intensive Care Units, patients undergoing surgery (especially gastrointestinal) and those with severe underlying disease who are subject to a prolonged hospital stay. However, the pre-eminent risk factor is the use of antibiotics (16,22). Nosocomial acquisition of *C. difficile* is a serious consideration for some institutions, particularly those with high inpatient populations, chemotherapy wards, or long-term patient care. The nosocomial diarrhoea caused by toxin-producing *C. difficile* is an important problem for both the clinicians and the infection control team in the hospitals, because of its epidemic spreading and the important cost implications. Since the 1980s, a number of studies have documented the nosocomial acquisition of *C. difficile.* Some hospital wards have a high rate of colonization by these microorganisms. Despite major efforts to control the spread of CDAD in healthcare facilities, this pathogen remains a problem worldwide that continues to be responsible for both endemic and epidemic nosocomial diarrhoea (14-24). *C. difficile* infections have become a considerable problem in most European countries, including Hungary.

1.2. Typing methods used with *C. difficile*

1.2.1. Phenotypic typing methods

The laboratory isolation and identification of nosocomial *C. difficile* strains have necessitated the development of typing methods to provide a better follow-up of the epidemiology of the disease and to afford a better insight into the pathogenicity of various strains. Numerous typing methods have been developed on the basis of various phenotypic and genotyping markers of this organism in order to understand the nosocomial epidemiology of *C. difficile* infection. Early methods were necessarily based on phenotypic properties such as antibiograms. In one of the first documented outbreak investigations, Burdon *et al.* in 1982 found a resistance pattern to 3 antibiotics in isolates from a surgical ward that were distinct from isolates in the rest of the hospital (19). However, this approach is at best only rudimentarly, and a more detailed approach was tried by Wüst *et al.*, who combined plasmid analysis, soluble protein polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis of extracellular antigens and antibiograms (32). Sell *et al.* used a combination of bacteriocin and bacteriophage typing methods, with only limited success, because a high percentage of strains are non-typeable (33). It has been shown that phage typing may be difficult to use because of the frequent lysogeny of *C. difficile* strains (34). Immunochemical fingerprinting of EDTA-treated cell extracts of *C. difficile* was evaulated by Poxton (35), and radio-PAGE of [^{35}S]methionine-labelled proteins by Tabaqchali (36). Nakamura *et al.* were the first investigators to use serum agglutination as a typing method by raising 3 antisera against *C. difficile* (37). Delmée's group improved this method and developed a serotyping scheme that could recognize 19 distinct serogroups (38). This method has frequently been used as the standard by which other typing methods are compared. Although serotyping and phage typing are simple and useful methods, only a few laboratories can perform them because these methods require the maintenance of stocks of sera and specific phages.

These early methods were ostensibily developed to understand the epidemiology of *C. difficile* infection at a local level. Many of these investigators found evidence that a single type was responsible for a number of cases within their hospital, thus confirming that *C. difficile* disease could be a cross-infection problem. There was a need for typing schemes that could be applied to further understanding of the epidemiology of *C. difficile* disease on a wider scale. Mulligan *et al.* found good a correlation between the types recognized by plasmid profiling, serotyping, PAGE of cell-surface antigens and immunoblotting (39). Sodium dodecylsulfate SDS-PAGE of whole-cell proteins was applied to investigate an outbreak, and this method yielded a maximum of approximately 40 bands, ranging in size

from 18 to 100 kDa. SDS-PAGE of EDTA-extracted cell surface antigens was compared with serogrouping by Ogunsola *et al.* (40). This method yielded bands between 30 and 67 kDa. Their results generally correlated well with the results of serogrouping, and could in fact differentiate between some members of the same serogroup. The whole-cell fingerprinting method of pyrolysis mass spectrometry (PMS) has been successfully used as a means of investigating putative *C. difficile* outbreaks (41, 42). This method has the advantage that it can cope with a large throughput of strains and has a high degree of discrimination. Its disadvantages, however, are the initial cost of the equipment and its inability to assign a permanent type to a strain.

1.2.2. Molecular typing methods

Molecular typing methods are generally regarded as superior to phenotypic methods in terms of the stability of expression and the greater degrees of typeability, and a number of methods have been applied to *C. difficile.* Plasmid profiling has proved largely unsuccessful, due to the sparse distribution of these extrachromosomal genetic elements within the species (43). Analysis of chromosomal DNA of *C. difficile* was attempted by Kuijper *et al.*, who used whole-cell DNA restriction endonuclease analysis (REA) with *Hind*III in an investigation which demonstrated cross-infection between 2 patients in the same room (44). Restriction fragment length polymorphism (RFLP) is an alternative genotypic method that involves initial REA digestion followed by gel electrophoresis and Southern blotting with selected labelled nucleic acid probes to highlight specific restriction site heterogenicity (45). The arbitrarily primed polymerase chain reaction (AP-PCR) is a genotypic method that permits the detection of polymorphism within the target genome without prior knowledge of the target nucleotide sequence (46). A closely related method, called random amplified polymorphic DNA (RAPD), commonly uses two oligonucleotide primers, which are short in length (cc. 10 bp) and also arbitrary in sequence. Barbut *et al.* evaluated a RAPD method using two 10 bp primers in an investigation of AAD in AIDS patients (47). PCR ribotyping uses specific primers complementary to sites within the RNA operon, it was first applied to *C. difficile* by Gurtler, who targeted the amplification process at the spacer region between the 16S and 23S rRNA regions (48). This part of the genome has been shown to be very heterogeneous, in contrast with the rRNA genes themselves, which are highly conserved. *C. difficile* has been demonstrated to possess multiple copies of the rRNA genes in its genome, which vary not only in number between strains, but also in size between different copies on the same genome. This method was improved by Cartwright *et al.*, who used the same primers as

Gurtler: their PCR fragments of similar size range could be separated by straightforward agarose gel electrophoresis instead of denaturing PAGE gels (49). Furthermore, they demonstrated that the banding patterns were not affected by the quantity of DNA used in the reaction (a problem associated with the AP-PCR and RAPD methods), and that the PCR ribotype marker was stable and its expression reproducible. This approach was adapted for routine use by O'Neill *et al.*, who improved the methodology even more by greatly simplifying the DNA extraction method (50). With the use of modified primers to the 16S-23S spacer region, this method produced amplicons ranging from 250 to 600 bp in length that could be separated by straightforward agarose gel electrophoresis. The discriminatory power was compared with Delmée's serogroups and gave different banding patterns for each of the 19 serogroups.

This method has been used routinely by the UK Anaerobe Reference Unit (ARU) in Cardiff: from over 3 000 strains that they have examined, a library consisting of 116 distinct ribotypes has been constructed to date (51). Pulsed-field gel electrophoresis (PFGE) allows the whole genome to be analysed after digestion with rare cutting restriction endonucleases, such as *Sma*I, *Ksp*I, *Sac*II or *Nru*I, which produce up to 10 fragment length polymorphisms per strain. PCR ribotyping was deemed more discriminatory than AP-PCR and PFGE methods in a study. Bidet *et al.* compared all 3 methods and concluded that PCR ribotyping, although marginally less discriminatory than PFGE, offered the best combination of advantages (52). Spigaglia *et al.* also found good correlations between PFGE and PCR ribotyping, but experienced 8 isolates that were non-typable by PFGE (53). Many workers have also noted, that some strains are repeatedly untypable by PFGE, due to degradation of the extracted DNA. Studies have shown that these PFGE-untypable strains belong in serogroup G, which corresponds to PCR ribotype 1 in the library of the Cardiff ARU. The toxinotyping method developed by Rupnik described 11 toxinotypes and has been extended to PCR ribotyping (54). Good correlations between the methods was noted, and toxinotyping to each type in the PCR library revealed 5 novel toxinotypes and ribotypes that had consistent changes in their toxin genes. A recently described alternative PCR target for typing purposes was the flagellin gene *flicC*, described by Tasteyre *et al.*; *flicC* could discriminate 9 different RFLP patterns (55).

All typing methods have certain advantages and disadvantages, but their ultimate contribution to knowledge is dictated by their performance according to the criteria listed by Struelens: namely, typeability, reproducibility, stability, discriminatory power and epidemiological concordance (56).

1.3. Biological place of the genus *Bacteroides sensu stricto*

1.3.1. Taxonomy, phylogeny

Bacteroides fragilis was originally observed and described as *Bacillus fragilis* by Veillon and Zuber in 1898 (57). In 1919, the species was transferred to the genus *Bacteroides* by Castellani and Chalmers (58). The other species that are currently members of the genus *Bacteroides sensu stricto* were described by Distaso (*B. thetaiotaomicron*), Eggert and Gagnon (*B. vulgatus*, *B. distasonis*, *B. ovatus* and *B. uniformis*), Holdeman and Moore (*B. eggerthii*) and Johnson *et al.* (*B. caccae*, *B. merdae* and *B. stercoris*) (59,60). The genus *Bacteroides sensu stricto* has undergone dynamic taxonomic changes in recent years and at present contains 12 species of anaerobic non-spore-forming, Gram-negative bacilli, with non-motile, rod-shaped cells 0.5 to 1.3 by 1.6 to 11 μm in size, formerly termed the *B. fragilis* group. Conventional methods that have been used to identify *Bacteroides* spp. are based upon phenotypic characteristics, including carbohydrate fermentation and other biochemical tests. However, phenotypic methods have an inherent risk of misidentification because of 1) the variable nature of some biochemical reactions and their dependence upon the environmental conditions, 2) the need for subjective interpretation of the results, 3) the fact that some related species differ by only one phenotypic reaction, and 4) the occurrence of "intermediate" organisms with characteristics inconsistent with those of recognized species. The invention of biochemical, chemical and, more recently, molecular biological techniques has done much to clarify the inter- and intrageneric relations of species classified in this genus. The procedures employed include:

- physiological characterization,
- electrophoretic patterns of dehydrogenases,
- fatty acid profiles,
- cellular lipid analysis,
- DNA-DNA hybridization,
- oligonucleotide cataloguing,
- 5S-16S rRNA sequence comparisons.

1.3.2. *Bacteroides* as members of the normal microflora

Members of the *Bacteroides* spp. are major constituents of the normal colonic microflora, and are also found in smaller numbers in the female genital tract, but are not common in the mouth or upper respiratery tract. The colon contains the largest total

populations of microorganisms of any inhabited region of the human body. The *Bacteroides* are the most numerous members of the normal microbiota in the lower intestine, representing nearly 10^{11} cells per gram of faeces (dry weight) (61). *B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis* are numerically dominant in the normal colonic flora, with only 1 to 5 % of the cultivable colonic bacteria comprising *B. fragilis*, although this figure has been challenged as being an underestimate.

1.3.3. Role of the genus *Bacteroides sensu stricto* in infection

Essentially all anaerobic infections emanate from the indigenous flora of the body. Rarely, as in "classical anaerobic" infections, such as in certain clostridial infections, they may be acquired exogenously. Four major sources of anaerobic bacteria are commonly associated with human infections: the oral cavity, the gastrointestinal tract, the genitourinary tract and the skin. Although anaerobic infections have been diagnosed in most anatomic sites of the human body, the vast of majority arise from the indigenous flora on mucosal surfaces, and to a less extent from the skin. Since virtually all these organisms are endogenous, there are always some predisposing factors for anaerobic infections. Factors leading to a reduced oxidation-reduction potential or oxygen tension, or both, produce an environment which is favourable for the growth of anaerobic bacteria (62). While the *Bacteroides* are the predominant members of the flora of the lower intestine, they are also opportunistic pathogens causing infections in body sites other than the large bowel, where they normally reside. These infections are primarily situated close to the colon. The *Bacteroides* are involved in *ca.* 80% of all human anaerobic infections, where *B. fragilis* is the most frequent causative agent, despite its low abundance in the intestine (63). To become pathogenic, the *Bacteroides* need a reduction in the defence mechanisms of the body and the synergistic action of other, usually aerobic or facultative anaerobic microbes. The most common infections associated with them are intraabdominal abscesses, peritonitis and wound infections of the large intestine. *Bacteroides* spp. are also the principal pathogens of the female genital tract and pelvic inflammatory abscesses and are implicated in anaerobic pulmonary abscesses. *B. fragilis* is frequently involved in brain abscesses secondary to chronic otitis media, and is a major pathogen in cases of diabetic foot ulcer syndrome. Although bacteraemia is commonly associated with *B. fragilis* infections, endocarditis caused by *Bacteroides* spp. is rare (64-69).

1.3.4. Pathogenicity factors

Most of the anaerobic bacteria are not overtly invasive, but they do have virulence mechanisms by which they can cause infections in certain circumstances (62,64). The circumstances in which anaerobic infections of the *Bacteroides* can be initiated are disruption of the mucosal wall of the intestine (surgery, perforated or gangrenous appendicitis, perforated ulcer, diverticulitis, tissue necrosis and inflammatory bowel disease), trauma, the presence of a foreign body, poor blood supply, corticosteroid and/or immunosuppressive therapy, immunosuppression, angiopathy, malignancy or other disorders, previous or concomitant proliferation of facultative anaerobic bacteria and diabetes mellitus. It has also been suggested that anaerobic bacteria inhibit phagocytosis, which would in turn lead to the further growth of anaerobic and facultative anaerobic organisms. Anaerobic bacteria can thus act synergistically with non-anaerobic bacteria in the mixed flora of anaerobic infections, or with each other. During anaerobic infections, the involved organisms may spread to adjacent tissues and localize into abscess. The most important virulence mechanisms of the genus *Bacteroides sensu stricto* are as follows (64):

- Capsule of *B. fragilis*
- Surface structures and adhesins
- Iron uptake mechanism
- Lipopolysaccharide (LPS) and stimulation of release of biological response modifiers
- Enterotoxin of *B. fragilis*
- Aerotolerance

1.3.5. Antibiotic resistance

Five groups of antimicrobial agents are active against most of the anaerobic bacteria of clinical importance. These are nitroimidazoles, such as metronidazole or tinidazole, carbapenems, such as imipenem or meropenem, or more recently faropenem, amphenicols such as chloramphenicol or thiamphenicol, combinations of β-lactam drugs with β-lactamase inhibitors (tazobactam, sulbactam or clavunic acid), and certain new quinolones such as trovafloxacin, clinafloxacin, moxyfloxacin, sitafloxacin and gemifloxacin. *B. fragilis* is more resistant to antimicrobial agents than most other anaerobic bacteria. Small numbers of *B. fragilis* strains are resistant to all the drugs mentioned above, except for the amphenicols (70, 71). Less active against anaerobic bacteria, but still useful, are cefoxitin, clindamycin, tetracyclines and broad-spectrum penicillins such as ticarcillin and piperacillin. 15-25% of the *Bacteroides* spp. are resistant to these compounds in many hospitals in the USA and

elsewhere. The actual resistance rate varies with the geographic location, but the general trend is in the direction of increasing resistance to all antimicrobial agents. Resistance to the least active antibiotics (most β-lactams and tetracyclines) was low or zero before the introduction of these drugs, and rose to the recent levels because of their continuous use. The main types of antibiotic resistance mechanisms are as follows: an altered affinity in target binding, a decreased permeability for the antibiotic, and the presence of an inactivating enzyme.

In general, the 5-nitroimidazoles (5-NI) and carbapenems are the classes of the most active antimicrobial agents against the *Bacteroides* spp. Strains of *Bacteroides* spp. with resistance to imipenem or metronidazole have been isolated, but only rarely.

1.3.5.1. Mechanisms of β-lactam resistance

The β-lactam antibiotics are the most varied and widely used of all antimicrobials. Resistance to the β-lactam agents can be caused by any of the 3 major resistance determinants: the production of β-lactamases, changes in penicillin-binding proteins (PBPs), and changes in outer membrane permeability to β-lactams. The most common mechanism is the production of β-lactamases. Efflux has recently been suggested as an additional source of resistance. The first β-lactam-resistant *Bacteroides* strains were observed as early as 1955. Production of the β-lactamases by anaerobes occurs commonly (72). In the *B. fragilis* spp., the current status of β-lactamase production as assessed by nitrocefin hydrolysis is 75-100% for *B. fragilis*, 90-100% for *B. ovatus*, about 100% for *B. vulgatus*, 91-100% for *B. thetaiotaomicron* and 58-85% for *B. distasonis* (73). Today, at least 34 β-lactamases originating from different clinical isolates, about 20 of which were isolated from anaerobic bacteria, have been described with unique aminoacid sequences or differentiated phenotypic behaviour. Many of these enzymes belong in closely related families with similar functions. These enzymes are most commonly found constitutively expressed, not induced by subinhibitory concentrations of antibiotics and chromosomally coded. An increasing proportion of strains produce a high level of β-lactamase, where insertion of an isertion sequence (IS) element may be responsible for the increased expression of the resistance genes (74-76). On the basis of a detailed characterization of these enzymes, *Bacteroides* β-lactamases can be assigned into 4 groups in the scheme developed by Bush *et al.* in 1995 (77). The most common β-lactamases fall into group 2e, with molecular masses of 30-40 kDa; they are inhibited by cloxacillin, pCMB, clavulanic acid, sulbactam and tazobactam, have acidic pIs, hydrolyse cephalosporins rather than penicillins, and do not hydrolyse cefoxitin, latamoxef or imipenem. The second type of β-lactamase of *Bacteroides* belongs in Bush group 2d. Only a few examples are known, among them *B. fragilis* GN11499, which is a

broad-spectrum β-lactamase that hydrolyses penicillins (including cloxacillin) and is inhibited by clavulanic acid and sulbactam. The third group of β-lactamases consists of enzymes that hydrolyse cefoxitin and moxalactam. The hydrolysis of these antibiotics by these enzymes is slow, but nevertheless contributes to resistance to these agents. They are inhibited by clavulanic acid, this property differentiating them from the fourth group of β-lactamases. Cefoxitin resistance has proved to be transferable via conjugation between the *Bacteroides* strains, and the resistance gene, *cfxA*, has been shown to reside on a conjugative transposon.

The fourth type of β-lactamases of *Bacteroides* are the metallo-β-lactamases, which are capable of hydrolysing cephamycins and carbapenems. The first example of these enzymes was described by Yotsui *et al.* in 1983 (78). A subsequent, more detailed characterization of one such enzyme from the latter publication showed that it was inhibited by EDTA, but not by clavulanic acid and sulbactam, and zinc ion reversed the inhibition by EDTA (79). A metallo-β-lactamase with a somewhat different substrate profile, but with a similar inhibition profile and similar physical characteristics, was described by Hedberg *et al.* (80). All these enzymes caused substantial resistance to imipenem (MICs of > 128 μg/ml) and had pI values in the range 4.5-5.2 and molecular masses of 25-33 kDa; they belong in Ambler's molecular class B and Bush group 3. Some clinical isolates of *B. fragilis* have been described that produce EDTA-sensitive and clavulanic acid-resistant β-lactamases, although these exhibit lower levels of resistance to imipenem, with MICs in the range 0.25-32 μg/ml. This type of resistance is characteristic of *B. fragilis*, but a few exceptions have been found. The genes of some of these carbapenemases from the USA and UK have been cloned and sequenced, or sequenced from a PCR fragment which demonstrated that these genes, *cfiA* or *ccrA*, are chromosomally located and highly homologous. Moreover, Khushi *et al.* reported that *cfiA*-positive *B. fragilis* strains were present before 1987, the year of introduction of imipenem (81). Conjugation studies revealed that imipenem resistance was not transferrable; still resistant clones could be selected after a one-step mutation at a frequency of *ca* 10^{-7} from suitable strains with an imipenem MIC =1 μg/ml and carrying a 'silent' carbapenemase gene. This mutation proved to be an insertion into the promoter region of the *cfiA* gene of an IS element, and the strong promoter carried by the IS element facilitated the high-level expression of the carbapenemase gene. The 'silent' *cfiA* genes can normally express only very low amounts of β-lactamases because of their weak promoters. The prevalence of *cfiA*-positive strains in France was estimated as 2.4% by hybridization with a *cfiA* internal probe, and 0.8% exhibited resistance to imipenem (82,83). In the UK, the prevalence of these strains was shown by PCR to be 6.9% and the rate of resistance was found to be 0.6%. The study by

Podgljen *et al.* also revealed that, despite the fact that the *cepA* and *cfiA*-carrying *B. fragilis* strains are indistinguishable by normal biochemical tests, they proved to belong in 2 separate groups by IS element content, ribotyping and AP-PCR-generated fragment pattern (84). This mutually exclusive grouping was hypothesized to correlate with the homology groups described by Johnson *et al.* (85). Comparison of small-subunit ribosomal DNA sequences of 16 strains of *B. fragilis* demonstrated that they separated into 2 subgroups, one in which the *cfiA* gene was carried and whose strains belonged in Johnson's homology group II, and one that consisted of *cfiA*-negative strains and whose strains belonged in Johnson's homology group I. Moreover, the *cfiA*-positive strains could be divided into 2 more subgroups, one of which gave strong hybridization signals with a *cfiA* probe under high-stringency conditions and one which hybridized only weakly. This difference might reflect the existence of 2, most likely closely-related *cfiA*-type genes. Similar results were found by Edwards and Greenwood (86), where carbapenemase-positive *B. fragilis* strains exhibited distinct LPS and OM profiles. While *cepA* and *cfiA* genes are not normally transferable, an exception was described by Bandoh *et al.*, who reported a strain of *B. fragilis* that contained a metallo-β-lactamase gene on a small plasmid transferable by conjugation (87).

PBPs are the targets of binding for β-lactam antibiotics. Upon binding, β-lactam antibiotics interfere with the peptidoglycan cell wall, leading to bacterial lysis. Thereby, binding to PBPs is the critical factor that determines whether a β-lactam agent will be effective as an antimicrobial agent. In *Bacteroides* strains, 3-5 PBPs exist: a PBP-1 complex with one of 3 different enzymes, PBP-2 and PBP-3. These PBPs are assumed to correspond to the essential high-molecular weight PBPs found in Gram-negative bacteria. There may be other low-molecular weight PBPs, but the number of these proteins varies among strains, thereby suggesting that they are probably not critical for growth.

Antibiotics must first reach their target to have an effect. Limited access of the antibiotic to its target is another type of antibiotic resistance mechanism. Examples of this type of mechanisms are the Gram-negative porins, which limit the diffusion of antibiotics through the outer membrane, and efflux pumps, which remove antibiotics from the bacterial cytoplasm. Mutants with a decreased expression of porins are not very resistant to certain anbtibiotics. This occurs because even the most effective permeability barrier of bacteria cannot completely shut out the influx of small molecules. To achieve significant levels of drug resistance, a second contributor is required in addition to a low permeability barrier. Decreased permeability has often been associated with increased β-lactamase production as a cause of resistance against β-lactam agents in Gram-negative bacteria. In certain *B. fragilis*

spp. strains, resistance has been found to be correlated with an alteration in the permeability barrier and the production of inactivating enzyme.

1.3.5.2. Mechanisms of 5-nitroimidazole resistance

The 5-NIs are imidazoles that contain a nitro group at position 5 of the imidazole ring. They are synthetic compounds which have various substituents on the aromatic ring, either on the nitrogen at position 1 or on the carbon at position 2, and such synthetic derivatives are commonly used as antimicrobial agents. The more common agents include: metronidazole, ornidazole, tinidazole and dimetronidazole (88). They are particularly useful in the prophylaxis and therapy of infections caused by the *Bacteroides* spp., since these bacteria are commonly resistant to many other antibiotics and there is a lack of significant side-effects with this therapy (89). The 5-NIs are prodrugs whose uptake and activation require intracellular reduction of the nitrogroup, resulting in the production of cytotoxic short-lived radicals. The redution process consists of 4 sequential monoelectronic steps leading to the formation of the 1) a nitroradical anion ($R\text{-}NO_2^{\bullet-}$), 2) a nitroso compound (R-N=O), and finally 3) a hydroxylamino derivate (R-NHOH) (90).

Resistance to metronidazole among the *Bacteroides* is rare, but may be increasing (91). In one group of cases, multiple mutations are responsible for the resistance, where isolates exhibit a reduced uptake of the drug, a reduced nitroreductase activity, and a decreased pyruvate, ferredoxin oxidoreductase activity in combination with an increased lactate dehydrogenase activity. This mechanism exerts high-level resistance and is not transferable. In the second group of cases, the MICs for metronidazole are lower than or near the standard breakpoint (16 μg/ml) and the MICs for tinidazole and ornidazole are higher than that for metronidazole. The mechanism causing the resistant phenotype of this group of strains has proved to be associated with the presence of resistance genes, which are carried on 3 plasmid types, pIP417 (7.7 kb), pIP419 (10 kb) and pIP21 (7.3 kb), and on the chromosome (*B. fragilis* strain BF8). The resistance genes, called *nimA* (pIP417), *nimC* (pIP419), *nimB* (BF8) and *nimD* (pIP421), have been cloned and sequenced and have *ca* 70% identity at the amino acid level (92,93). The fifth resistance gene, *nim*E, was reported in 2000 by Stubbs *et al.*, and was found in 5 metronidazole-resistant (MICs >32 μg/ml) *Bacteroides* strains. *nim*E exhibited 75% DNA sequence similarity with the closest *nim*B gene (94). The *nim* genes are preceded in all cases by IS elements, which upregulate them in a mechanism like that for imipenem resistance. The *nim* genes encode a nitroimidazole reductase protein and this type of resistance is transferable by conjugation. A PCR-based method for the detection of *nim* genes was evaluated by Trinh and Reysset (95).

2. AIMS

1. To examine the prevalence of toxin-producing *C. difficile* in faecal samples of inpatients who suffer from diarrhoea during their hospital stay in a 1200-bed university hospital in Hungary. To evaluate the ordering patterns of Hungarian physicians for the *C. difficile* toxin-determination tests and their impact on the laboratory results and therefore on the clinical diagnosis, results obtained on stool specimens for which *C. difficile* had been specifically requested were compared with those for which it had not.

2. To collect clinically relevant isolates of *C. difficile* from different hospital wards in the same hospital, in order 1) to determine whether these isolates are genetically similar to *C. difficile* isolates collected in the UK by the ARU in Cardiff; and 2) to identify the most common ribotypes of *C. difficile* in our hospital, in Szeged.

3. To determine the prevalence of the *nim* resistance genes among clinical isolates from *Bacteroides* spp. in Hungary and to characterize the different types of *nim* genes.

4. To determine the presence of the *cfiA* gene by means of PCR in clinical isolates and the normal faecal flora of *B. fragilis* collected in Hungary.

5. To use a PCR-RFLP technique for the reliable identification of clinically important *Bacteroides* strains at the species level.

3. MATERIALS AND METHODS

3.1. Epidemiological investigation of *C. difficile*

3.1.1. Specimens

During a one-year period (from April 1999 to March 2000), 3 081 faecal samples were screened for bacterial enteric pathogens. The samples originated from different hospital wards of the University Hospital of Szeged. Two different groups of samples were investigated for the presence of *C. difficile* toxin: the first group consisted of samples where clinicians had specifically requested the investigation of *C. difficile* toxin (n=375). In these cases, the presence of toxin A was determined by using the VIDAS (bioMérieux, France) toxin detection kit. The second group consisted of 570 stool samples which were selected by different selection criteria in the laboratory in order to investigate the presence of the "free toxin" in the faeces by use of a cytotoxin assay. The selection criteria were: long-stay hospitalization (>5 days), loose, liquid stools (bloody or/and mucoid), lack of other enteric pathogenic bacteria, viruses, ova or parasites, and from the clinicians for no request *C. difficile* toxin investigation (96).

3.1.2. Toxin testing

3.1.2.1. Cytotoxicity testing

Cytotoxin assay by tissue culture: Toxin B has a potent effect on the cytoskeletal or microfilament structure of mammalian tissue culture cell lines. Freshly-taken faecal samples were used for the standard procedure for the cytotoxin assay, using the HeLa cell line. A suspension (1:1) was prepared from the faeces with phosphate-buffered-saline (PBS, pH 7.4). After centrifugation (3 000 g, 30 min) the supernatant was filtered through a 0.22 μM low protein-binding membrane filter (MILLEX®-GV) and the filtrates were stored at –20 °C until the detection of the *C. difficile* toxin by the cytotoxin assay which, was performed once a week. Before use, the filtrates were diluted 1:10; 1:20; 1:40 and 1:80. 10 μl of each dilution was inoculated onto a HeLa cell monolayer, which was examined after an overnight incubation and again after 48 h. The *C. difficile* VPI 10463 strain was used as a positive control. To confirm the specificity, any cytopathic effects was compared with a negative PBS control, and neutralization with *C. difficile* goat antitoxin was always carried out. Non-specific cytopathic effects may result from viruses or enterotoxins of other organisms, such as *C. perfringens* (97,98).

3.1.2.2. VIDAS, *C. difficile* Toxin A II method

VIDAS *C. difficile* Toxin A II (CDA2) (bioMérieux, France) is an automatized test for the detection of *C. difficile* toxin A, using the ELFA technique (enzyme-linked fluorescent assay). The solid-phase receptacle (SPR) serves as a solid phase, and as the pipetting device for the assay. In the first step of the test, the sample is cycled in and out of the SPR for a specified length of time. Toxin A present in the sample will bind to the rabbit anti-toxin A antibody coating the interior of the SPR. Unbound sample components are washed away. A mouse monoclonal anti-*C. difficile* toxin antibody conjugated with alkaline phosphatase is cycled in and out of the SPR and will bind to any toxin A bound to the SPR wall. A fluorescent substrate, 4-methylumbelliferyl phosphate, is introduced into the SPR. Enzyme remaining on the SPR wall will catalyse the conversion of the substrate to the fluorescent product 4-methylumbelliferone. The intensity of fluorescence is measured by the optical scanner in the instrument; this intensity is proportional to the amount of toxin in the sample. The assay is completed in approximately 60 min, and the test value and the interpretation appear on the results sheet. Interpretation according to the test value is as follows: negative, equivocal or positive. Samples with an equivocal result within this test should be tested with the confirmatory assay, using the original processed sample or a freshly processed aliquot of the specimen. The sample in the block strip is mixed with a goat anti-toxin antibody for a specified length of time, while the sample in the reference strip is mixed with normal goat serum for the same time. A mouse monoclonal anti-*C. difficile* toxin A antibody conjugated with alkaline phosphatase is cycled in and out of the solid phase, following by the usual washing steps. If toxin A is present in the sample, the goat anti-toxin A antibody will bind to the toxin in the block strip sample well, thereby reducing the amount of toxin which can bind to the rabbit anti-toxin A antibody coating the interior of the SPR. The normal goat serum in the reference strip sample well will have no effect on the toxin. Thus, when toxin A is present in the sample, the block strip relative fluorescence value (RFV) should be reduced by at least 80% as compared with the reference strip RFV. The advantage of this method is that the results are reported to the clinicians within 2 h. For those specimens where the physicians had requested the detection of *C. difficile* toxin, the VIDAS *C. difficile* Toxin A II (CDA2) (bioMérieux, France) system was used (99).

3.2. PCR ribotyping of clinical isolates of *C. difficile*

3.2.1. Bacterial strains

Stool samples (n=252) of 252 inpatients with diarrhoea (loose stools, 3x4/day, bloody and/or mucoid) from the University Hospital in Szeged were cultured for *C. difficile* during a 3-month period. Other bacterial and/or viral enteric pathogens were not isolated from these stool samples in this period. A few extraintestinal *C. difficile* isolates were also included in the study, originating from a range of extraintestinal sites during the same period. Out of the 68 *C. difficile* isolates used in this study, 57 strains were isolated from diarrhoeal faecal samples and 8 originated from other clinical materials. Three reference strains were included as controls: *C. difficile* NCTC 11382 (toxin A-positive, toxin B-positive), *C. difficile* CCUG 20309 (toxin A-negative, toxin B-positive) and *C. difficile* NCTC 11206 (toxin A-negative, toxin B-negative). Stool samples were cultured on selective CCFA (Wadsworth Manual) as soon as possible in the laboratory, and incubated at 37 °C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon USA) for 24-48 h. Other clinical materials were cultured on prereduced Columbia agar base (Oxoid, United Kingdom) supplemented with 5% cattle blood, vitamin K_1 and haemin, and incubated at 37 °C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon, USA) for 24-48 h. The identification of the isolates was based on the characteristic colonial morphology, green-yellow UV fluorescence (under UV light) and latex agglutination (MicroScreen *C. difficile* Latex Slide Agglutination Test; Mercia Diagnostics, UK).

3.2.2. PCR ribotyping method

Isolates were cultured anaerobically overnight under anaerobic conditions on Fastidious Anaerobic Agar (FAA; LabM, Bury, UK) supplemented with 6% horse blood. Approximately 10 colonies were picked, and crude template nucleic acid was prepared by the resuspension of cells in 5% (wt/vol) Chelex-100 (Bio-Rad, Hemel Hempstead, United Kingdom) and boiled for 12 min. After the removal of cellular debris by centrifugation (15,000 x g for 10 min), 10 µl of supernatant was added to 90 µl of PCR mix. The PCR mixture contained 50 pmol of each primer, 2U of Taq polymerase (Pharmacia) and 2.25 mM $MgCl_2$. Reaction mixtures were subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. Amplification products were concentrated to a final volume of 25 µl by heating at 75 °C for 90 min before electrophoresis. Electrophoresis was carried out at 100 mA and 200 V in 3% Metaphor agarose (FMC Bioproducts, Rockland, Maine) for 4.5 h, at 8 °C. Products were visualized by staining the gel for 20 min in ethidium bromide (0.5 µg/ml). To allow normalization of all gel patterns, a

molecular size standard (100 bp; Advanced Biotechnologies, Epsom, United Kingdom) was run at 5-lane intervals (50,51).

3.2.3. Primers

PCR ribotyping uses specific primers complementary to the 3` end of the 16S rRNA gene and the 5' end of the 23S rRNA gene to amplify the variable-length intergenic spacer region. The oligonucleotide primers used were 5'-CTG GGG TGA AGT CGT AAC AAG G-3' (positions 1445 to 1466 of the 16S rRNA gene) and 5'-GCG CCC TTT GTA GCT TGA CC-3' (positions 20 to 1 of the 23rRNA gene) (50,51).

3.2.4. Analysis of banding patterns and library construction

Gel images were analysed with GelCompar image analysis software (version 4.0; Applied Maths, Kortrijk, Belgium). The criterion for the proposal of a new library type was the existence of clearly discernible, reproducible differences in PCR ribotype pattern from those of all other existing types.

3.3. Detection and characterization of *nim* genes of *Bacteroides* spp.

3.3.1. Bacterial strains

A total of 202 non-duplicate *Bacteroides* strains were collected from the different hospital wards of the 1200-bed University Hospital in Szeged during two years (2000 and 2001). *Bacteroides* strains were cultured on prereduced Columbia agar base (Oxoid, United Kingdom) supplemented with 5% cattle blood, vitamin K_1 and haemin, and incubated at 37°C, under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon, USA) for 48 h. Routine phenotyping identification was carried out with the ATB ID 32A kit (bioMérieux, S.A., Marcy l'Etoile, France). Quantitative determination of the metronidazole resistance of the strains was performed with E-test strips according to the manufacturer's instructions (AB Biodisk, Solna, Sweden) on Columbia agar.

3.3.2. *nim* gene PCR

The presence of *nim* genes in 18 strains that exhibited reduced sensitivity to metronidazole, and 1 strain which was high-level resistant to metronidazole, was assessed by PCR with the primers NIM-3 and NIM-5, according to the methods described previously (95). Positive control strains containing *nim* genes included *B. fragilis* BF8 (*nimB*), *B. fragilis* 638R containing plasmid pIP417 (*nimA*), *B. fragilis* 638R containing plasmid pIP419 (*nimC*), and *B. fragilis* 638R containing plasmid pIP421 (*nimD*). *B. fragilis* Kw 388/1 was used as a *nimE*

control strain (confirmed previously by a Southern hybridization method) and *B. fragilis* NCTC 11295 was included as a *nim* gene-negative control.

PCR products were resolved by agarose (1.5%) gel electrophoresis with a molecular weight standard (100 bp; Advanced Biotechnologies, Epsom, UK), stained with ethidium bromide (0.5 μg/ml) and visualized with UV light.

3.3.3. RFLP analysis

Amplification products from *nim* gene were treated with the restriction endonucleases *Hpa*II and *Taq*I, according to the manufacturer's instructions (Promega). Digestion products were resolved in Metaphor agarose (3.5%) (FMC Bioproducts) at 100 V in TAE (40 mM Tris-acetate buffer, 1 mM EDTA, pH 8.0) for 2.5 h and visualized under UV light after staining for 20 min with ethidium bromide (0.5 μg/ml) (94).

3.3.4. Detection and mapping of the activating IS elements

The presence of the *IS1186* element was determined with the primer pairs *IS1186A* and *IS1186B*, using a cycling parameters as follows: starting denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, at 48 °C for 1 min, at 72 °C for 1 min and at 72 °C for 10 min as final elongation. The *IS1186* elements were mapped upstream of the *nimA,B* genes, using the NIM-5 and IS1186A primers, where the following cycling parameters were applied: at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, at 50 °C for 1 min, at 72 °C for 3 min and at 72 °C for 10 min as a final elongation step. The *B. fragilis* strain 638R carrying a pIP417 plasmid was used as a positive control in both experiments. The PCR products were detected in 0.8% agarose gels in TBE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.26) containing 0.5 μg/ml ethidium bromide and visualized as above along a 100 bp-3 kb ladder fragment preparation (Fermentas, Vilnius, Lithuania).

3.3.5. Plasmid isolation and Southern hybridization

Plasmid DNA was isolated as described before (100), except that chloramphenicol was not added, and electrophoretized as described in the previous section. For molecular weight determination, as *Escherichia coli* V517 plasmid preparation was used. DNA blotting to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, UK) was performed as in [15], while the *nim*A PCR fragment labelling was carried out with the Gene Images Random Prime labelling kit (Amersham Pharmacia Biotech) as recommended by the supplier, and hybridization as recommended in the random prime labelling kit leaflet of the same company. Hybridized probes were detected with the CDP-Star detection kit (Amersham Pharmacia Biotech).

3.4. Detection of the carbapenemase gene (*cfiA*) among *Bacteroides* spp.

3.4.1. Bacterial strains and plasmids

During the period 1998-2000, a total of 242 *Bacteroides* spp. isolates were studied by PCR for the presence of the carbapenem resistance determinant, the *cfiA* gene. Out of 242 strains, 231 were randomly selected clinically significant isolates, 1 *B. fragilis* isolate originated from a prostatic-abscess of a dog, and 10 isolates were obtained from faecal samples from healthy subjects. Isolates were identified by conventional biochemical tests or with the Rapid ID 32A system (bioMerieux). *B. fragilis* NCTC 9344 (carbapenem-sensitive) and *B. fragilis* TAL 3636 (a metallo-β-lactamase producer) were included as controls. The pJST241 plasmid, carrying the cloned *cfiA* gene, was obtained from M.H. Malamy. The Qiagen mini plasmid purification kit (Qiagen Inc., Hilden, Germany) was used for plasmid isolation.

3.4.2. Antibiotic resistance determination

The MIC values of carbapenems were determined by the Etest (AB Biodisk, Stockholm, Sweden) on brain heart infusion (BHI) agar supplemented with yeast extract (5 g/l), haemin (5 mg/l) and menadione (1 mg/l) according to the instructions of the manufacturer. MICs were read after incubation for 48 h in an anaerobic environment (Bactron, Shell Lab., Cornelius, USA).

3.4.3. Determination of the β-lactamase and specific imipenemase activities

β-lactamase activity was determined quantitatively with nitrocefin (0.10 mM) in 50 mM sodium phosphate buffer (pH 7.0, 37 °C) by a spectrophotometric method. One unit of β-lactamase was defined as the amount which formed 1.0 μM of product per min under the given conditions. The specific imipenemase activities were determined by following the change in absorbance at 299 nm of a mixture of bacterial sonicate (0.2 ml), imipenem (0.2 ml, 250 mg/l) and phosphate buffer (pH 7.0, 0.6 ml), and the results were given in nmole hydrolysed imipenem/mg protein/ml (101).

3.4.4. Investigation of outer membrane (OM) proteins

OM proteins of selected *B. fragilis* strains were examined by a method based on that described by Carlone *et al.* (102). Bacteria from cultures grown in 20 ml of BHI broth were washed and resuspended in 1 ml of PBS. The sonicated cells were centrifuged to remove intact cells. Each supernatant was mixed with sodium N-lauroylsarcosinate to give a final concentration of 2% (w/v), and incubated at room temperature for 30 min. After centrifugation at 12 000 rpm for 20 min, the pellet was washed twice in PBS. The OM

proteins were solubilised by boiling with 50 µl of Laemmli sample buffer for 5 min and separated by SDS-PAGE.

3.4.5. Detection of *cfi*A gene by PCR

The PCR method was used to screen the isolates for the *cfiA* gene. Bacterial cells grown on the surface of BHI agar plates were suspended in water and boiled for 10 min. The supernatants of the centrifuged suspensions (2 min, 10 000 rpm) were used as template DNA. Reaction mixtures each contained 5 µl of 10x reaction buffer (Sigma or USB), 1 µl (2.5 mM) each of dATP, dCTP, dGTP and dTTP (Sigma), 1 µl (35 pmoles) of each primer, 5 µl of template DNA, 33 µl of sterile water and 1U of Taq polymerase (Sigma). The primers had the nucleotide 557 to 582 sequence 5'-TCC ATG CTT TTC CCT GTC GCA GTT AT-3' and the complementary 1266 to 1285 sequence 5'-GGG CTA TGG CTT TGA AGT GC-3' (82). The reaction mixtures were incubated for 40 cycles in a programmable heating block (GeneAmp, PCR System 9600, Perkin Elmer, Norwalk, USA) for 1 min at 92 °C, 2 min at 50 °C and 2 min at 72 °C, with a final extension of 10 min. PCR products were visualized on agarose gels containing ethidium bromide under UV light, and their sizes were compared with those of a molecular weight marker (100 bp DNA ladder, Sigma) and the product generated from *B. fragilis* TAL3636 as a positive control.

Southern blotting with a *cfi*A-specific probe obtained from the PCR product of the pJST241 plasmid was used to confirm the PCR results. PCR products were resolved on 1% agarose gels and were transferred to Nylon membranes (Amersham, UK) by capillary transfer. Radioactive labelling of the probe was performed with the Amersham Megaprime random priming kit (Amersham, UK) under the conditions recommended by the supplier. Prehybridization was carried out at 65 °C for 1 h, with hybridization at 65 °C overnight in 5xSSC, 5xDenhardt's solution containing 0.5% SDS and 100 µg/ml salmon sperm DNA. Radioactive labelled probe DNA with an activity of *ca* 10^7 cpm was added to the hybridization solution after prehybridization. The filters were washed as follows: twice in 2xSSC, 0.1% SDS at 65 °C for 15 min and twice in 0.2xSSC, 0.1% SDS at 65 °C for 15 min. Damp filters were packed in Saran Wrap and were exposed to X-ray films at -70 °C with an intensifying screen.

3.4.6. Nucleotide sequencing of PCR products

Before nucleotide sequencing, the amplified PCR products were ethanol-precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of absolute ethanol at room temperature to remove unincorporated primers. DNA sequencing was performed with an automated sequencer (ABPrism model 373, Applied Biosystems, Inc.) with the AmpliTaq FS

DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The obtained DNA sequence was compared with those of known *Bacteroides* carbapenemase genes with the BLAST client programme accessible from the Internet at the National Center for Biotechnology Information.

3.5. 16S rDNA PCR-RFLP analysis of *Bacteroides* spp.

3.5.1. Bacterial culturing and identification of strains

Bacterial strains were cultured on FAA (Lab M, Bury, UK) supplemented with 5% (vol/vol) horse blood in an anaerobic atmosphere (10% CO_2 and 10% H_2 and N_2) at 37 °C. A total of 96 well-characterized strains, including the type strain of each species, were analysed: *B. fragilis* (24 strains), *B. thetaiotaomicon* (15 strains), *B. vulgatus* (12 strains), *B. distasonis* (10 strains), *B. ovatus* (6 strains), *B. uniformis* (5 strains), *B. caccae* (4 strains), *B. merdae* (2 strains), *B. splanchnicus* (2 strains), *B. stercoris* (2 strains), *B. variabilis* (1 strain) and *B. eggerthii* (1 strain). A further 4 strains from different clinical samples that gave ambiguous results in phenotypic tests were analysed. Control strains were obtained from the National Collection of Type Cultures (NCTC) and the American Type Collection (ATCC). Routine phenotyping identification was carried out with the commercially available ATB API 20A kit (bioMérieux, France) and conventional biochemical methods according to the instructions of the VPI Manual (103).

3.5.2. 16S rDNA PCR method

Crude template nucleic acid was prepared with GeneReleaser (Cambio, Cambridge, UK). A single colony harvested after overnight culturing on FAA was resuspended in 20 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). GeneReleaser (20 µl) was added, vortex-mixed for 20 s, and treated for 6 min at full power in a microwave owen. (750 W) before centrifugation (12, 000 x g for 2 min). Template nucleic acid in the supernatants was stored at –20 °C.

16S rDNA genes were amplified with the universal primers pA (5'-AGA GGT TTG ATC CTG GCT CAG; positions 8 to 27) and pH (5'-AAA GGG AAG GGT GGG ATC CAA GCC CGC A; positions 1540 to 1520). Template nucleic acid (5 µl) was included in a 50 µl PCR reaction mixture (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, gelatin 0.01%, 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each primer, and 1U of *Taq* DNA polymerase [Promega, Madison, Wisconsin, USA]). Reaction mixtures were denatured for 3 min at 95°C and subjected to 30 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 1 min, and polymerization at 72 °C for 90 s (94,95).

PCR products were resolved by agarose (1.5%) gel electrophoresis with a molecular weight standard (100 bp, Advanced Biotechnologies, Epsom UK), stained with ethidium bromide (0.5 μg/ml), and visualized with UV light.

3.5.3. RFLP analysis

Amplification products from 16S rDNA PCR were treated with the restriction endonucleases *Hpa*II and *Taq*I, according to the manufacturer's instructions (Promega). Digestion products were resolved in Metaphor agarose 3% [wt/vol], FMC Bioproducts) at 100 V in TAE (40 mM Tris-acetate buffer, 1 mM EDTA, pH 8.0) for 2.5 h and visualized with UV light after staining for 20 min with ethidium bromide (0.5 μg/ml). To allow normalization of RFLP patterns, a molecular weight standard (100 bp, Advanced Biotechnologies, Epsom, UK) was run at 5-line intervals. Restriction profiles were analysed with GelCompar (Applied Maths, Kortjik, Belgium) (94).

4. RESULTS

4.1. Prevalence of *C. difficile* diarrhoea in inpatients (Papers III, IV and IX)

During the study period (from April 1999 to March 2000), 3 081 faecal samples were screened for common bacterial enteric pathogens. All of these samples were from adult inpatients. 63 *Salmonella* (2.05%) and 4 *Campylobacter* (0.13%) isolates were found in the faecal samples of 67 patients (Table 3). No isolates of other enteropathogenic bacteria (*Yersinia* sp., *Shigella* sp. or enteropathogenic *E. coli*) were found during this period.

Table 3: Prevalence of common enteric bacterial pathogens in stool samples of inpatients between 1 April 1999 and 1 April 2000

Total number of samples	3 081
Number of positive samples	67 (2.2%)
Salmonella sp.	63 (2.05%)
Shigella sp.	0
Yersinia sp.	0
Campylobacter sp.	4 (0.13%)
Enteropathogenic *E. coli*	0

A total of 945 stool samples were investigated for the presence of *C. difficile* toxin, and 178 of them (18.9%) were toxin positive with one of the 2 toxin-detection methods. None of the stools which were positive for *C. difficile* toxin contained other enteric pathogenic bacteria, virus or parasites. The 178 *C. Difficile* toxin-positive stool samples originated from 178 adult inpatients (99 males, 79 females, average age: 44.3 years +/- 21.1). Only for 375 of the 945 stool samples obtained from the diarrhoeal patients had the clinicians requested *C. difficile* toxin testing, and 58 (18.3%) were toxin A-positive. Of the remaining 570 samples, which fitted the selection criteria, 120 (21.05%) were toxin-positive by the cytotoxin assay. To assess the usefulness of routine laboratory screening for *C. difficile* toxin in the faeces of diarrhoeal inpatients undergoing a prolonged stay in hospital versus testing only on request, the data were analysed according to the wards of origin (Table 4).

Table 4: Incidence of *C. difficile*-toxin positivity among diarrhoeal patients in various hospital wards between 1 April 1999 and 1 April 2000

Hospital wards	Samples tested	
	On request (n= 375)	After laboratory selection (n =570)
	No. of samples/No. of positives (%)	
Surgery	22/7 (32%)	50/17 (34%)
ICU with surgical profile	14/2 (14%)	46/11 (24%)
Internal Medicine	50/12 (24%)	156/37 (24%)
Haematology	164/18 (11%)	107/17 (16%)
Gynaecocology	2/0 (0%)	11/2 (18%)
Urology	3/0 (0%)	19/8 (42%)
Others	120/19 (16%)	181/28 (16%)
Altogether	375/58 (18%)	570/120 (21%)

Most samples tested were from the Haematological Department. The overall positivity was 13%, but 17 patients would have been missed if only requested specimens had been tested for *C. difficile* toxin. The highest percentage positivity was observed among patients in the surgical ward. In both groups of specimens, more than 30% proved to be toxin-positive, but the number of requested investigations was much lower than the number of specimens selected by the laboratory criteria (22 *vs* 50). No positive species were found among the 3 samples requested from the Urological Department, but 42% of the samples selected for the testing of *C. difficile* toxin according to the criteria set up by the laboratory proved to be positive. The difference observed in toxin-positivity rates between the two examined groups was also significant in the case of the Intensive Care Unit with a surgical profile (14% positivity in the requested group *vs.* 24% in the laboratory-selected group). Of 120 stool specimens submitted with a specific request for *C. difficile* toxin testing from other (Orthopaedic, Traumatologic, Oncological, Neurological, Nephrological, *etc.*) hospital wards 19 (16%) were toxin-positive. Twenty-eight (16%) of the 181 specimens from these wards for which *C. difficile* toxin testing was not requested were also cytotoxin-positive.

Only in the case of 93 patients was it possible to find out retrospectively what antibiotics were prescribed by the physician and used at the time of the sampling and/or 1 month previously (Table 5).

Table 5: Distribution of *C. difficile* toxin–positive patients according to the antibiotic therapy prior to the onset of diarrhoea

Antibiotics	Number of patients
β-lactam antibiotics	39
Quinolones	10
Doxycycline	3
Glycopeptides	2
Metronidazole	2
Fluconazole	8*
Antibiotic combination (including β-lactams)	37 (29)
Altogether	93

*only in combination with antibiotics

Out of 93 patients, 37 had received antibiotic combinations and 56 patients had received monotherapy. Most of the patients had been treated with β-lactam antibiotics either in monotherapy (39 cases) or in combination with other antibiotics (29 cases). The quinolones were the second most frequently used antibiotic group in connection with *C. difficile*-associated diarrhoea in our study. Tetracycline was administered to 3 patients, glycopeptides to 2 patients and nitroimidazoles to 2 patients. The antifungals administered were fluconazole (8 cases) and amphotericin B (1 case). In all cases, the antifungal drugs were given together with other antibiotics.

4.2. Results of PCR ribotyping of *C. difficile* (Paper VIII)

This was a retrospective study involving the molecular characterization of case isolates to delineate endemic strains in our hospital. Analysis of the PCR ribotyping method was performed on a total of 57 faecal isolates from the symptomatic patients in different clinical units. Typing was also carried out on 8 background strains selected from clinical cases. The sources, toxigenicity profiles and PCR ribotypes of the isolates and control strains are listed in Table 6. All of the isolates originated from different patients in various wards at the University Hospital of Szeged. Most of them were hospitalized in the Departments of Surgery, Haematology, Internal Medicine and the Intensive Care Units; only a few patients were treated in the Departments of Cardiac Surgery and Ophthalmology. None of them were outpatients.

Table 6: Origin of strains from the University Hospital of Szeged and their PCR ribotyping

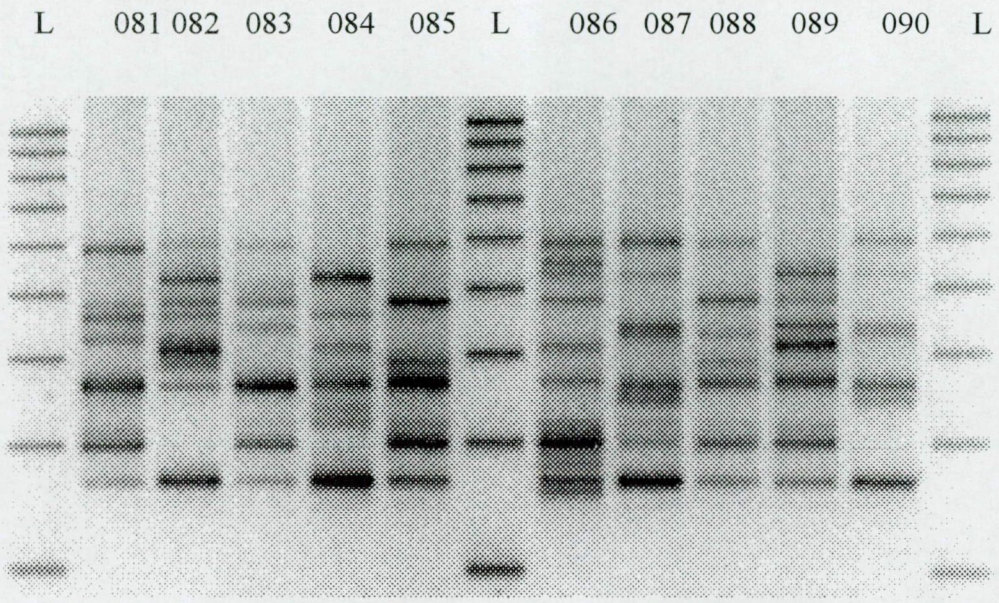
Origin of strains			No. of strains belonging to PCR ribotype														
Ward	Clinical material	No. of strains	001	002	009	010	012	015	032	040	068	070	087	092	114	124	New
Internal Medicine	Faeces	17	2		1		3			1	1		8		1		
Paediatrics	Faeces	17	5		1	1	3		1				4			1	1
Surgery	Faeces	6		1	2							1	2				
	Drain	3					1						1				1
	Wound	1											1				
	Bile	1	1														
Haematology	Faeces	5							1				3			1	
Dermatology	Faeces	1											1				
Ophthalmology	Conjun.	1				1											
Neurology	Faeces	1											1				
Cardiac Surgery	Faeces	1					1										
Intensive Care U.	Faeces	9					4						4	1			
	Wound	1					1										
	Intraabd.	1						1									

Conjunc.: conjunctiva; intraabd.: intraabdominal

Control strains NCTC 11382, CCUG 20309 and NCTC 11206 belonged to PCR ribotypes 043, 036, and 038, respectively.

In vitro toxin testing of the isolates revealed that there were both toxigenic and non-toxigenic isolates in the samples. During a period of 3 months, 57 *C. difficile* strains were isolated from 252 faecal samples; 44 of them (77%) were toxin-producing, as detected by using an immunoassay for the detection of *C. difficile* toxin A and cytotoxicity on the Vero cell line (toxin B). Six of the 8 clinical samples other than faeces collected during the same period (75%) were toxin-producing. Toxin A-negative, toxin B-positive strains were not isolated from these samples during this period. All investigated *C. difficile* strains were susceptible to vancomycin and metronidazole. The PCR ribotypes consisted of patterns comprising 3-12 bands, with the size of the bands varying from approximately 250 to 600 bp (Fig 2).

Fig. 2: PCR ribotype profiles obtained with *C. difficile* isolates, ribotypes from 81 to 90 (control strains from ARU collection)



A total of 15 different ribotypes were detected among the 65 isolates tested (Table 7).

Table 7: Distribution of PCR ribotypes among Hungarian *C. difficile* strains

PCR	Reference ribotype strain*	No. (%) of strains	Production of toxin A	Production of toxin B	Serogroup**	Toxino-type of the reference strain#
001	R8366	8(12.3)	+	+	G	0
002	R8375	1 (1.5)	+	+	A2	0
009	R8269	4 (6.3)	-	-	I	
010	R8270	2 (3.0)	-	-	D,E6	
012	R6187(S51)	13 (20.0)	+	+	C,A,G,K	0
015	R6685(S38)	1 (1.5)	+	+	G	0
032	R6598	2 (3.0)	-	-	ut	
040	R100917	1 (1.5)	-	-	ut	
068	IS56	1 (1.5)	-	-	ut	
070	R9367	1 (1.5)	+	+	K	I
087	R11840	25 (39.0)	+	+	G	0
092	R10630	1 (1.5)	+	+	ut	0
114	R11212	1 (1.5)	-	-	ut	
124	R11919	2 (3.0)	-	-	ut	
New type	-	2 (3.0)	-	-	nt	

ut.: untypable, nt.: not tested.

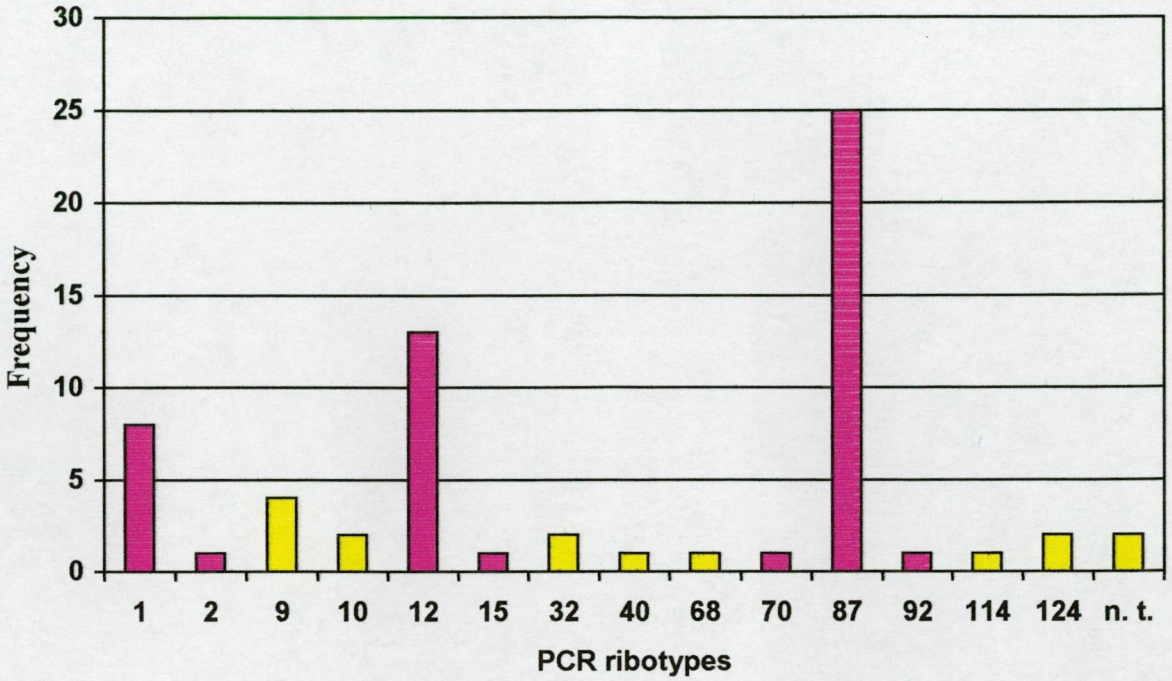
* ARU Reference Strain

**Serogroup of the reference strains has been made to Delmee serotype (38).

Toxinotype of the reference strains was performed according to methods described previously (54).

The 50 (77%) toxigenic isolates could be classified into 7 visually distinct ribotypes, and the 15 non-toxigenic isolates into 8 PCR ribotypes. Of the 50 toxigenic isolates tested, 46 (92%) belonged in just 3 PCR ribotypes (087, 012 and 001), with type 087 being the most common, accounting for 50% (25 of 50) of the toxigenic isolates tested. The remaining 4 isolates (8%) belonged in 4 other ribotypes. There was a wider distribution among the ribotypes of non-toxigenic strains. A total of 15 toxin-negative isolates were investigated in this study, and ribotype 009 was isolated most frequently (4/15) (Fig. 3). 2 non-toxigenic isolates belonging in ribotype 010 were isolated from conjunctiva and newborn faeces. This PCR ribotype is frequently isolated from the faeces of neonates and small children in the UK and other countries.

Fig. 3: Distribution of PCR ribotypes among Hungarian *C. difficile* strains

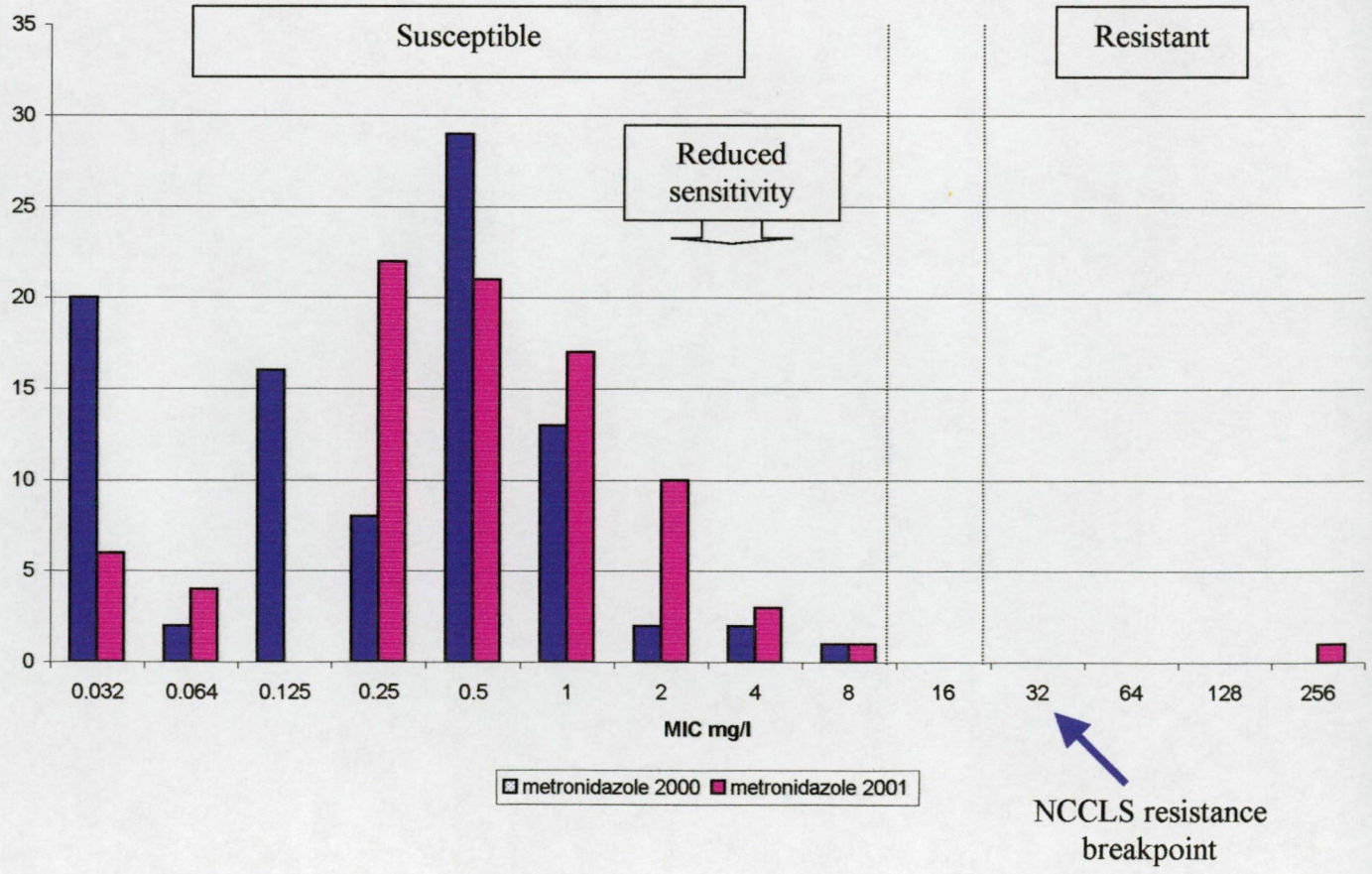


There was no significant correlation between the PCR ribotypes and the origin of the isolates investigated in this study. Some of the *C. difficile* isolates originating from different wards of the University Hospital of Szeged in Hungary belong in the same ribotype as *C. difficile* isolates collected in the UK by the ARU in Cardiff. To date, of the 3000 isolates of *C. difficile* typed by this method, 116 distinct PCR ribotypes have been recognized; a representative strain of each PCR ribotype has been stored in the ARU, Cardiff, UK (51). All but 2 of the investigated isolates could be typed by this PCR ribotyping method; the exceptions were non-toxigenic isolates (one from faeces, and the other from intraabdominal drainage). These 2 isolates exhibited the same pattern, which was distinct from those of all the ribotypes described previously, suggesting that it is a new type. More investigations are required to classify it with the cluster correlation algorithm. The integrity of the library was tested with control *C. difficile* strains. *C. difficile* NCTC 11382 was used as a toxigenic control strain, it belongs in ribotype 043. The non-toxigenic control strain was *C. difficile* NCTC 11206, which belongs in PCR ribotype 038, and the control toxin A-negative, toxin B-positive strain, CCUG 20309, belongs in ribotype 036. Strains within the library have also been analysed by other typing schemes; the PCR ribotyping method correlates with other typing schemes and allows subtyping of many of the types produced by other methods.

4.3. Prevalence and characterization of *nim* genes of *Bacteroides* spp. (Papers VII and X)

Susceptibility studies have documented the emergence of antimicrobial resistance among the members of the *Bacteroides* spp. and indicated differences in resistance patterns related to individual hospitals, geographic regions and antibiotic-prescribing regimens. A total of 202 non-duplicate isolates of *Bacteroides* spp. were isolated from different patients in various wards at the University Hospital of Szeged during the 2-year period. *B. fragilis* was the most commonly isolated species, constituting almost half of the total isolates referred. *B. thetaiotaomicron* was the second most common species, followed by *B. uniformis B. ovatus, B. vulgatus B. distasonis* and *B. merdae.* Less commonly seen were *B. caccae, B. eggerthii* and *B. stercoris.* All of them, except one high-level metronidazole-resistant strain, were susceptible to metronidazole (NCCLS resistance breakpoint 32 μg/ml) (104), but 4 of them (2.4%) in 2000 and 14 (7%) in 2001 exhibited reduced sensitivity (Fig. 4). The MIC_{90} was 1.0 μg/ml in both years.

Fig. 4: Distribution of metronidazole MIC values among the *Bacteroides* strains isolated in 2000 and 2001



All of the strains investigated by *nim* PCR were resistant to penicillin (MIC >32 μg/ml), but except for 2 strains (originating from an atherosclerotic wound: *B. fragilis* 29877/1, and an appendicular abscess *B. fragilis* 20384), all of them were susceptible to the other tested anti-anaerobic antimicrobial agents. The strain *B. fragilis* 29877/1 was highly resistant to amoxicillin/clavulanic acid (MIC >32 μg/ml), clindamycin (MIC >256 μg/ml) and cefoxitin (MIC 16 μg/ml), but despite the fact that this strain was susceptible to imipenem (MIC 0.125 μg/ml)), it carried a 'silent' *cfiA* gene as well. The strain *B. fragilis* 20384 was highly resistant to metronidazole (MIC >256 μg/ml), borderline-susceptible to cefoxitin (MIC 16 μg/ml) and susceptible to amoxicillin/clavulanic acid (MIC 2.0 μg/ml) and clindamycin (MIC 1.5 μg/ml).

Table 8: MICs of metronidazole and types of *nim* gene present for control and clinical *Bacteroides* strains

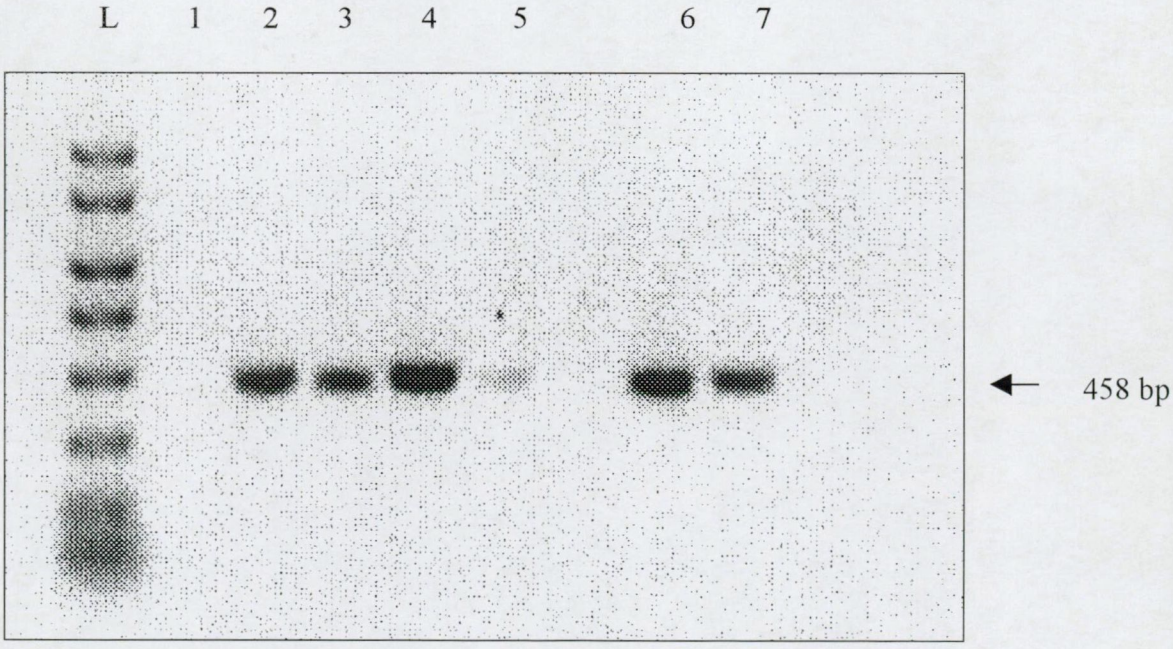
	Isolate	Number	Metronidazole MIC μg/ml	Presence of *nim* by PCR	Type of *nim* gene by RFLP
Positive	*B. fragilis*	638R (pIP417)	24	+	A
controls	*B. fragilis*	BF8	>32	+	B
	B. fragilis	638R (pIP419)	6	+	C
	B. fragilis	638R (pIP421)	16	+	D
	B. fragilis	Kw 388/1*	>256	+	E
Neg. control	*B. fragilis*	NCTC 11295	>32	-	-
Our isolates	*B. fragilis*	19924	12	+	A
	B. fragilis	29877/1	4	+	B
	B. fragilis	20384	>256	+	E

* confirmed by Southern hybridization.

The control strains containing the 5 *nim* genes all gave good visible PCR product with a length 458 bp. Of the 19 clinical strains analysed, 3 gave PCR product with primers NIM-3 and NIM-5 (Fig. 5). No PCR product was found for the other 16 strains which showed reduced sensitivity to metronidazole. The 5 different *nim* gene PCR products from the control strains produced unique digestion profiles with *Hpa*II and *Taq*I (Fig. 6/A,B). PCR products from *nim* genes in 3 clinical strains were identified by comparison of the digestion patterns with those from the 5 *nim* genes from the control strains. One of them (*B. fragilis* 19924), originating from intraabdominal pus, that gave a metronidazole MIC of 12 μg/ml, had a PCR

RFLP profile consistent with *nimA*, and the other strain (*B. fragilis* 29877/1) with a metronidazole MIC of 4 μg/ml, had a PCR RFLP profile consistent with *nimB* gene. The third strain (*B. fragilis* 20384), isolated from an appendicular abscess of a 2 year-old boy in 2001, gave a high-level metronidazole MIC (>256 μg/ml) and had a PCR RFLP profile consistent with *nimE* (Table 8). In addition to the *nim* gene types, the presence of the activating IS elements was also determined (80). With *IS1186* specific primers, the *IS1186* element was demonstrated in the genomes of the *nim*-positive strains; then, with one forward primer of the *IS1186* element (IS1186A primer) and a reverse primer from the *nim* genes (NIM-5), the localization of the *IS1186* elements was proved to be close enough and in correct orientation upstream of the *nimA* and *nimB* genes to activate them both in moderately-resistant *B. fragilis* strains. The plasmid profiles of the 2 *nim*-positive strains were also examined by the method used previously in our laboratory (105). In the strain *B. fragilis* 19924, a single plasmid of 7.7 kb was detected, while in the strains *B. fragilis* 29877/1 and 20384 (harbouring *nimB* and *nimE*, respectively), no plasmid could be found, during Southern hybridization of the non-radioactively labelled *nimA* probe hybridised to the 7.7 kb plasmid from *B. fragilis* 19924.

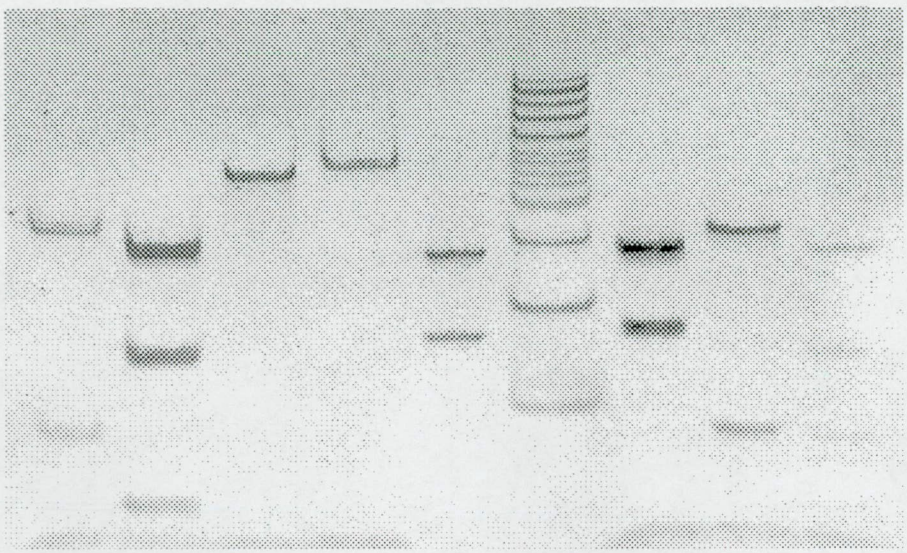
Fig. 5: *nim* PCR products of *B. fragilis* strains with elevated metronidazole MICs



L: molecular weight marker, 100 bp ladder
1: negative control
2–5: *nim* controls A, B, C and D, respectively
6: *B. fragilis* 19924 7: *B. fragilis* 29877/1

Fig. 6/A: PCR RFLP typing of *nim* PCR products with *TaqI* enzyme

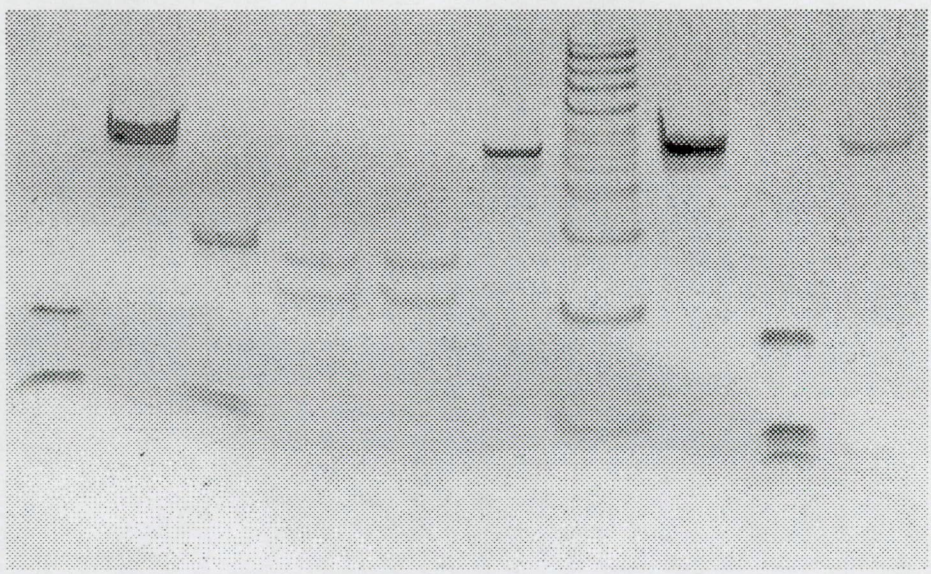
1. 2. 3. 4. 5. 6. 7. 8. 9.



1-5: *nimA-E* controls, respectively
6: 100 bp ladder
7: *B. fragilis* 20384 (*nimE*)
8: *B. fragilis* 19924 (*nimA*)
9: *B. fragilis* 29877 (*nimB*)

Fig. 6/B: PCR RFLP typing of *nim* PCR products with *HpaII* enzyme

1. 2. 3. 4. 5. 6. 7. 8. 9. 10.



1-3: *nimA-C* controls, respectively
4-5: *nimD*, 6 *nimE* controls
6: 100 bp ladder
7: *B. fragilis* 20384 (*nimE*)
8: *B. fragilis* 19924 (*nimA*)
9: *B. fragilis* 29877 (*nimB*)

4.4. Prevalence of *cfiA* gene of *Bacteroides* spp. (Papers I, II, V, VI and VII)

No expressed resistance to carbapenems was detected during the previous Hungarian resistance surveillances between 1987 and 1997, published by our laboratory (106,107). During the period 1998-2000, a total of 242 *Bacteroides* isolates were studied by PCR for the presence of the carbapenemase determinant, the *cfiA* gene. 2 *B. fragilis* isolates were included (both were isolated in 2000) which were found to be highly resistant to imipenem (MICs >256 μg/ml) and meropenem (MICs >32 μg/ml) by susceptibility testing. One of them was isolated from a gangrenous appendix of a 22-year-old male patient (*B. fragilis* 9259/5), while other *B. fragilis* isolate (515/2), which was found to be highly resistant to imipenem, originated from a prostatic abscess in a 9-year-old male German shepherd dog.

A 729 bp *cfiA* gene fragment was detected in 5 carbapenem-sensitive *B. fragilis* isolates (MICs 0.06-1.5 μg/ml), in 2 imipenem-resistant strains, and in none of the non-*fragilis* isolates by PCR. Out of the 5 *cfiA*-positive, but carbapenem-sensitive isolates, 4 had very low carbapenem MICs (0.06-0.25 μg/ml) (Table 9), with almost no differences between imipenem and meropenem. Very low β-lactamase activities were measured for these isolates. PAGE analysis of the OM proteins of the *B. fragilis* isolates with elevated MICs and those that were fully susceptible, but harboured the *cfiA* gene, did not reveal differences. The amplification of the regions upstream of the *cfiA* genes showed that no insertion occurred for the 5 sensitive isolates harbouring the 'silent' *cfiA* genes (amplification products of *ca* 300 bp found). The presence of 5 well-known IS elements (IS*942*, IS*1186*, IS*4351*, IS*1169* and IS*1170*) in the genome of these *cfiA*-positive isolates was also investigated. No IS was detected among the carbapenem-sensitive strains, with one exception (*B. fragilis* 29877/1), which is moderately metronidazole-resistant and contains IS*1186*. Southern blotting of the PCR products revealed homology to the probe from pJST241 plasmid (*cfiA*) in 3 isolates (*B. fragilis* isolates 20, 72 and 98). The PCR product of *B. fragilis* isolate 22 did not hybridize. Sequence analysis of the PCR product of *B. fragilis* isolate 22 showed that the 729 bp fragment bears *ca* 96% homology to the known *cfiA* genes.

In the cases of the 2 resistant strains, the molecular background was further analysed together with the specific imipenemase activities. DNA upstream of the *cfiA* in these strains was amplified by PCR: the primer upstream of the gene was derived from a conserved region, oligonucleotide G, and the downstream primer comprised the complementary sequence 565-598 within *cfiA*. Gel electrophoresis revealed upstream PCR products of 1.8 kb with these resistant strains. This suggested the insertion of an element of *ca* 1.5 kb upstream of the *cfiA* gene in both of the resistant strains. The presence of IS*942* (1.59 kb) was detected in the

genome in these strains, by PCR with IS element-type specific primers. The orientation and position of IS*942* relative to *cfiA* were examined by PCR mapping. A PCR product of 2.2 kb was obtained, identical to that exhibited by *B. fragilis* TAL3636, which indicated the "correct" orientation of IS*942* and its position upstream and adjacent to *cfiA*. This showed that IS*942* had the potential to act as a functional promoter in these isolates. The β-lactamase and specific imipenemase production of the *cfiA*-positive isolates were determined (Table 9). 4 of the 5 isolates with low imipenem MICs produced low amounts of β-lactamases, and the 2 resistant isolates produced high amounts of carbapenemase.

These results show that care should be taken in Hungary too as regards the emergence of carbapenem-resistant *Bacteroides* strains, because the prevalence of 'silent' strains is relatively high and imipenem-resistant strains could arise. Moreover, the expressed resistant cases should befollow up because of therapeutic considerations.

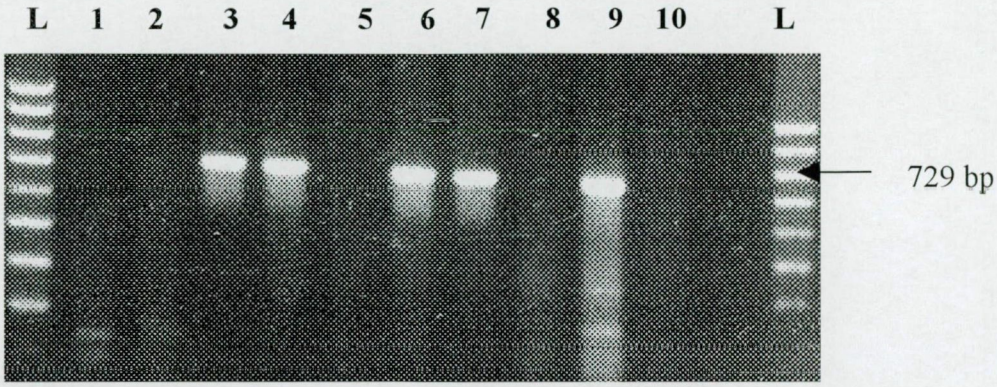
Table 9: Carbapenem resistance levels, ß-lactamase activities, and IS elements of *cfiA* gene positive isolates

Isolate	MIC (μg/ml)		β-lactamase-activity (U/ml)	Presence of *cfi*A	IS element upstream of *cfi*A
	Imipenem	Meropenem			
B. fragilis 22	0.06	0.125	0.0175	+	-
B. fragilis 20	0.06	0.06	0.0028	+	-
B. fragilis 98	0.125	0.25	0.0004	+	-
B. fragilis 72	0.25	0.25	0.0109	+	-
B. fragilis 29877/1	0.125	1.5	n. t.	+	IS*1186*
B. fragilis 9259/5	>256	>32	37.03*	+	IS*942*
B. fragilis 515/2	>256	>32	21.60*	+	IS*942*

n. t.: not tested

*specific imipenemase activities expressed in nmole hydrolysed imipenem/mg protein/min.

Fig. 7: *cfiA* PCR products of the 'silent' *B. fragilis* strains



1-8: Clinical isolates of *B. fragilis* 47, 39, 22, 20, 92, 72, 98 and 66, respectively

L: molecular weight marker, 9: *B. fragilis* TAL3636 + control, 10: *B. fragilis* NCTC 9344 –control

4.5. 16S rDNA PCR-RFLP analysis of *Bacteroides* spp.

Routine phenotypic identification:

All strains were non-motile, obligate anaerobic, Gram-negative bacilli. They were also strongly saccharolytic and bile-resistant and produced succinic acid as a major end-product of fermentation. The results of the biochemical tests used for the differentiation of the strains are shown in Table 10.

Table 10: Distinguishing phenotypic characteristics of *Bacteroides* spp. (94)

Species	No. of strains tested	Results with:									
		Indole	α-Fucosid	Arabin	Glycog	Melibi	Rhamn	Salicin	Sucr	Treh	Xyl
B. caccae	4	-	+	+	+	+	V	-	+	+	-
B. distasonis	10	-	-	V	+	+	+	+	+	+	-
B. eggerthii	1	+	-	+	+	-	+	-	-	-	+
B. fragilis	24	-	+	-	+	+	-	-	+	-	-
B. merdae	2	-	-	-	+	+	+	+	+	+	-
B. ovatus	6	+	+	+	+	+	+	+	+	+	+
B. stercoris	2	+	+	-	+	-	+	-	+	-	-
B. splanchnicus	2	+	+	V	-	V	-	-	-	-	-
B. thetaiotaomicron	15	+	+	+	+	+	+	V	+	+	-
B. uniformis	5	+	+	+	+	+	V	+	+	-	-
B. variabilis	1	+	+	+	+	+	+	+	+	-	+
B. vulgatus	12	-	+	+	+	+	+	-	+	-	v

+ positive, - negative, V: variable (103)

All but 2 of the type strains were identified correctly by the conventional biochemical methods. The type strains of *B. merdae* and *B. distasonis* could not be distinguished reliably

by routine phenotypic methods because larch arabinogalactan is no longer available. Of the 84 clinical strains analysed, 59 (70%) were identified unequivocally, 21 (25%) gave weak reactions for some critical biochemical tests (weak indole reactions, and variable fermentation reactions) and were identified only on repeat testing, and a further 4 (5%) could not be assigned reliably to a recognized species.

16S rDNA PCR-RFLP identification:

UPGMA dendograms of the 16S rDNA PCR restriction profiles obtained for type strains of *Bacteroides* spp. with *Hpa*II and *Taq*I are shown in Fig. 8. The *Hpa*II profiles allowed the 12 investigated type strains to be differentiated into 8 groups, while *Taq*I differentiated them into 11 groups. All the type strains could be separated by using the two-enzyme strategy, and comparison with the reference profiles permitted unequivocal identification of 81 (96.5%) of the 84 clinical strains (Fig. 9).

Fig. 8/A: UPGMA dendograms of *Hpa*II 16S rDNA PCR-RFLP profiles obtained with the control type strains of *Bacteroides* spp. (94)

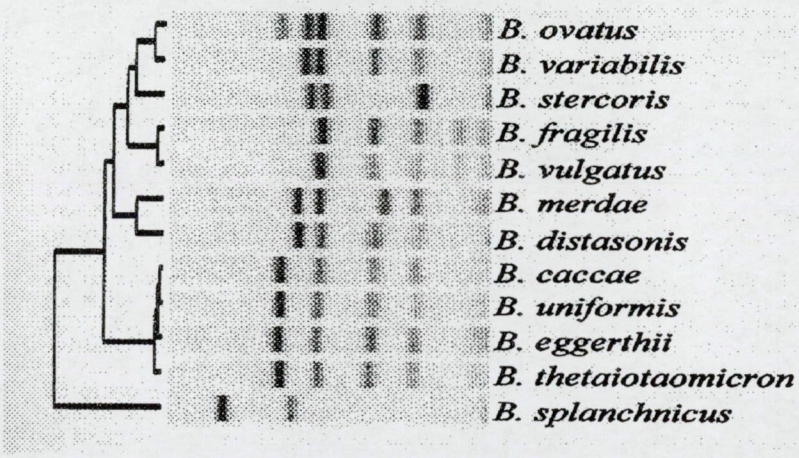


Fig. 8/B: UPGMA dendograms of *Taq*I 16S rDNA PCR-RFLP profiles obtained with the control type strains of *Bacteroides* spp. (94)

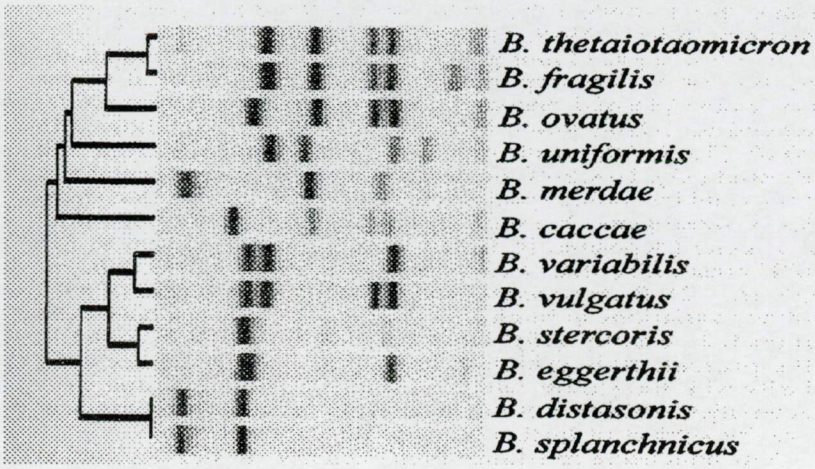


Fig. 9/A: *Hpa*II 16S rDNA PCR-RFLP profiles of investigated clinical isolates of *Bacteroides* spp.

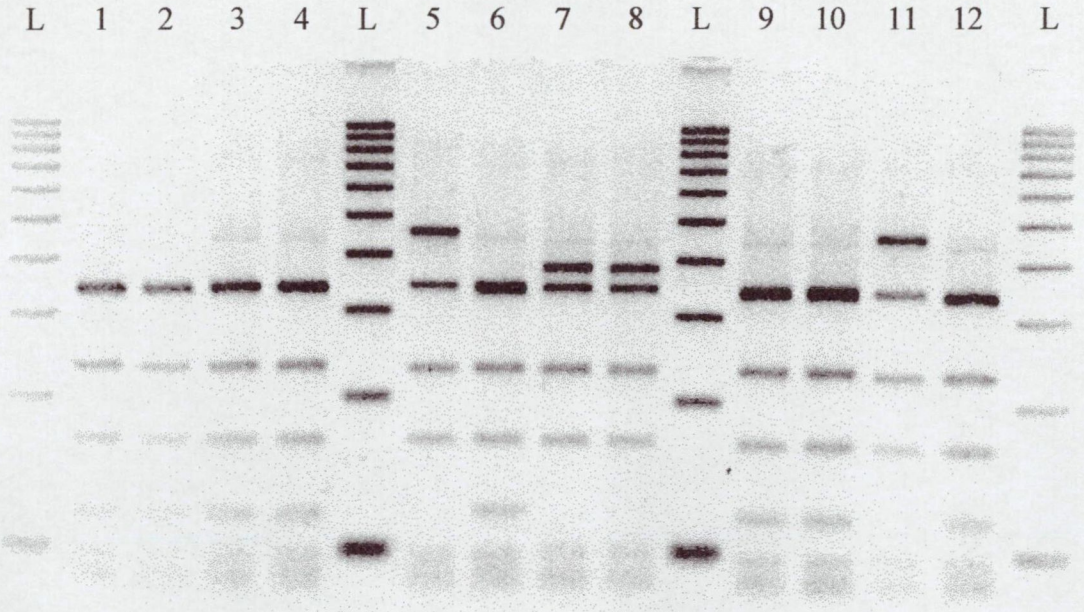
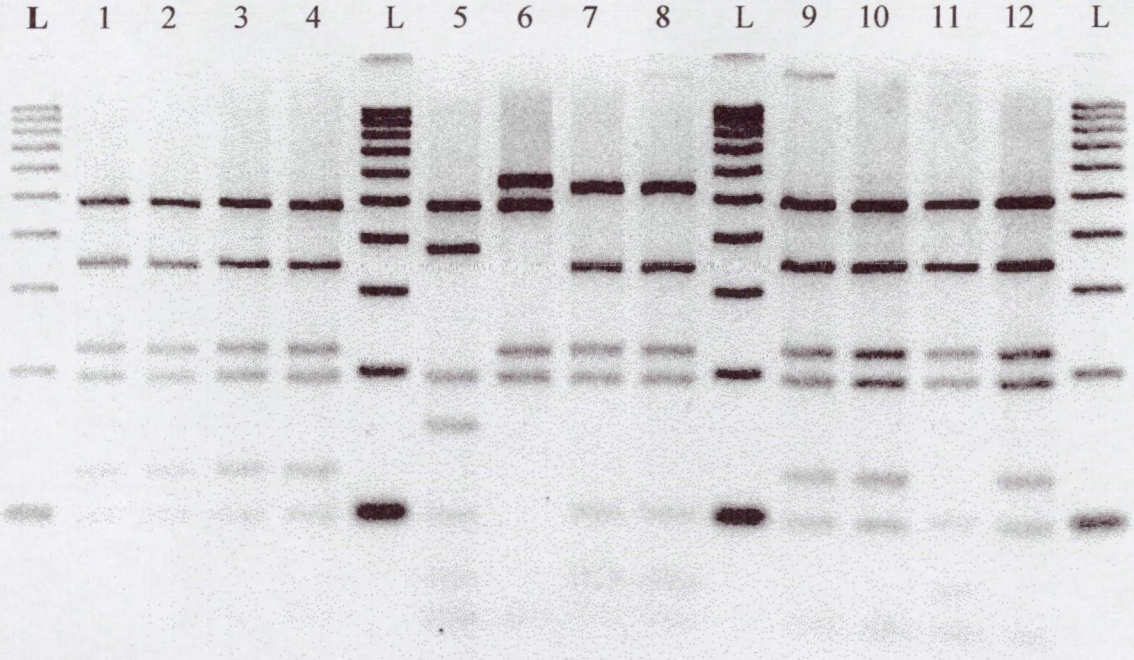


Fig. 9/B: *Taq*I 16S rDNA PCR-RFLP profiles of investigated clinical isolates of *Bacteroides* spp.



1-4, 9, 10, 12: *B. fragilis* 5: *B. uniformis*, 6: *B. vulgatus*, 7, 8: *B. ovatus*, 11: *B. thetaiotaomicron,* L: molecular weight ladder

Out of 8 strains which were not identified correctly phenotypically because of the weak biochemical reactions, 6 were identified as *B. ovatus,* 1 as *B. thetaiotaomicron,* and 1 as *B. distasonis* by 16S rDNA PCR-RFLP identification.

5. DISCUSSION

5.1. Evaluation of the prevalence of toxin-producing *C. difficile* in hospital-acquired diarrhoea

The normal bacterial flora in the gut provides an ecological barrier against significant colonization by pathogenic organisms (this protective effect of the normal microbiota is frequently referred as "colonization resistance"). Disruption of this protection by antibiotic use may permit the overgrowth of endogenous or nosocomially acquired pathogens such as *C. difficile.* The nosocomial acquisition of *C. difficile* is a serious consideration for some institutions, particularly those with high inpatient populations in chemotherapy wards, involving long-term patient care. During the hospital stay, the acquisition of *C. difficile* occurs frequently, even in non-epidemic situations, approaching a figure of 21% according to the data of Johnson (16). Risk factors associated with acquisition of the toxin-producing *C. difficile* include advanced age, severe underlying disease, antibiotic usage and length of hospital stay (20). According to some studies, by the fourth week of hospital stay, over half of the patients are likely to be culture-positive (17-21). The frequency of *C. difficile* as the aetiologic agent of nosocomial diarrhoea is not well known in Hungary. Because of the economic situation, microbiological laboratories do not carry out costly *C. difficile* toxin detection on inpatients with diarrhoea on a routine basis, unless it is specifically requested by the physician. Therefore, it is very difficult to evaluate the true prevalence of *C. difficile* diarrhoea in our hospitals. In our laboratory, we have found a significant number of *C. difficile* toxin-positive stools as compared with the presence of other enteric pathogens, such as *Salmonella* sp. and *Campylobacter* sp. *etc*, routinely tested in stool specimens. During a one-year study period, only for 375 stool samples obtained from diarrhoeal patients did the clinicians request *C. difficile* toxin testing, and 58 (18.3%) were toxin-positive. Of the 570 remaining samples, which were selected by the laboratory a further 120 (21.1%) were toxin-positive. There was no significant difference in toxin-positivity rate (18.0% *vs* 21.1%) between patients for whom *C. difficile* toxin testing was specifically requested and those for whom it was not. The overall positivity was 18.8% for those who became diarrhoeal during their stay in the hospital. 120 of the 178 patients who had *C. difficile* toxin-positive stool specimens would have gone unnoticed if toxin testing had been carried out only for those for whom the physicians had requested it. Most of the toxin-positive patients were treated with β-lactam antibiotics, either in monotherapy or in combination, before the onset of the diarrhoea. This Hungarian survey should draw the attention of physicians to the role of *C. difficile* as a

major nosocomial enteric pathogen in inpatients who have undergone antimicrobial treatment and/or surgery (especially gastrointestinal) and who have been subjected to a prolonged hospital stay. Our results support the finding of Bowman and Riley (28) that infectious diarrhoea in hospitalized patients is more likely to be caused by *C. difficile* than by any other enteric pathogen, and laboratories should therefore include its investigation for their routine.

5.2. PCR ribotyping of *C. difficile* isolates obtained in our hospital

PCR ribotyping has recently been proposed as an effective means of studying *C. difficile* epidemiologically. The Public Health Laboratory Systems ARU in Cardiff routinely uses this method. They typed over 3000 strains and constructed a library consisting of 116 distinct ribotypes. In total, 54 different PCR ribotypes have been identified from hospitalized patients to date. Type 001 accounts for 68% of the total of all hospital patient isolates in England and Wales. The International Typing Study (involving 7 groups of experts from the UK, Belgium, Australia and the USA), organized by Brazier in 1997, revealed that certain types were common to each typing method, indicating the distribution of the same types in hospitals in these countries (24,28). The prevalence of these ribotypes in Britain is in contrast with findings from some other European countries. There are only a few publications in the literature concerning the genetic relationship of pathogenic strains of *C. difficile* from various parts of the world, and especially regarding the Eastern European countries. In Eastern Europe, there has been only one study, in a Polish maternity hospital, where AP-PCR and PCR ribotyping methods were used. All environmental isolates and 11 of 31 neonatal isolates were of a single type, Type 1. There have been attempts to establish some form of standardization in the nomenclatures ascribed to strains typed by the various study groups. At present, these various types are uncoordinated and there is a lack of understanding as to how types relate to one another. The Polish ribotyping results have not been compared with the ARU reference library, and it has not been determined which PCR ribotype corresponds to their Type 1 (108). In our study, we compared the ribotypes of 65 *C. difficile* isolates originating from patients in Szeged, Hungary, with the library of *C. difficile* ribotypes in the ARU in Cardiff, UK. Although this sample size is small, the isolates originating from Hungarian inpatients display a very different distribution of PCR ribotypes as compared with those found by the ARU in the UK. The most predominant ribotype in the Hungarian survey of 65 isolates was PCR ribotype 087, a toxigenic type, which accounted for 39% of all isolates. The prevalence of this ribotype in Hungary is in contrast with the findings in other countries: this type was not common in England and Wales, where only 8 isolates of this

type were found among several thousand investigated isolates. We found 2 toxin non-producing isolates of a previously unrecognized type. We did not observe a significant correlation between the distribution of the PCR ribotypes and the origin of the strains during this period. Although no data have been published on the prevalence of *C. difficile* infection or the epidemiology of *C. difficile*-associated diarrhoea in Hungary, the isolation of toxigenic *C. difficile* from hospitalized patients suggests that this pathogen is responsible for certain cases of diarrhoea of undiagnosed origin and validates our efforts to establish its significance in Hungary.

5.3. First detection of metronidazole resistance in *Bacteroides* spp. in Hungary

Metronidazole is the first drug of choice for the empirical coverage of anaerobic infections. 5-NI resistance in Gram-negative anaerobes and clostridia has remained rare, despite several decades of usage. The first *B. fragilis* strain resistant to metronidazole was reported by Ingham *et al.* in 1978 (67). The articles published during the 1980s revealed only a few cases of *B. fragilis* that were resistant to metronidazole, and investigations into resistant strains and transmission mechanisms of resistance are reported only rarely. However, the emergence of metronidazole resistance in *Bacteroides* spp. in France (109), the UK (91,110), Kuwait (111), India (112), South Africa (113) and Morocco (114) has recently been described. The true incidence of metronidazole resistance in Hungary is probably underestimated, since the antimicrobial sensitivity testing of anaerobes is not performed routinely in most routine laboratories. Diagnostic laboratories must also take note of the existence of artefactual resistance to metronidazole of *Bacteroides* spp., false resistance associated with susceptibility testing under suboptimal anaerobic conditions. Monitoring of the susceptibility of clinical isolates of anaerobes, preferably by MIC determination under well-controlled anaerobic conditions, is necessary as one means of assessing the situation. In a previous study, none of the clinical isolates belonging in the family *Bacteriodaceae* that were obtained during a 10-year period (1987-1997) were found to be resistant to metronidazole in Hungary (106,107). During the present study, we detected the first high-level metronidazole-resistant strain and the first intermediate-resistant strains harbouring the metronidazole resistance determinant, the *nim* gene, in Hungary. A total of 202 *Bacteroides* spp. were investigated: PCR products from *nim* genes were identified in 3 clinical strains (1.5%); one of them held *nimA* and the other the *nimB* gene, also harbouring the upstream copies of the *IS1186/IS1168* element, a situation similar to that for the strains isolated and characterized previously in France. A novel *nim* gene (*nim*E) was present in a high-level

metronidazole-resistant (MIC >256 μg/ml) *B. fragilis* strain originating from an appendicular abscess. However, the novel *nim* gene exhibited only 75% DNA sequence similarity with the closest *nim* gene (*nim*B) (94). The origin and characterization of this novel nitroimidazole resistance determinant are the subject of further study in the anaerobe reference laboratories. 5-NI resistance genes were not detected in 16 further moderately metronidazole-resistant clinical strains (MICs 2-8 μg/ml). It is possible that other alternative mechanisms of resistance, such as that of NCTC strain 11295, are involved in these strains, or additional *nim* genes may exist that were not recognized by the PCR primers used.

The extent of metronidazole resistance in *Bacteroides* spp. is a most important issue, with profound implications for treatment. The presence of moderately metronidazole-resistant *Bacteroides* is difficult to detect by conventional susceptibility testing methods. We therefore need to continue to survey the antibiotic resistance among clinical isolates of *Bacteroides* spp. and screen for the presence of *nim* resistance genes.

5.4. Evaluation of the carbapenemase gene (*cfiA*) among clinical isolates of *Bacteroides* spp. in Hungary

In recent years, no imipenem-resistant isolates have been reported in Hungary (the MIC_{90} was 0.5 μg/ml in 1992 and 1 μg/ml in 1992-93) (107), despite the fact that imipenem or meropenem is used for treatment increasingly in severe infections involving aerobic and anaerobic bacteria. In these surveillances, the resistance breakpoint suggested by the NCCLS was used. Low rates of resistance to imipenem among *B. fragilis* strains have been found in France (115) and the USA (116), whereas in Japan (117), where imipenem is widely used, 2.4% 4.5% and 10.5%, respectively, of the *B. fragilis*, *B. thetaiotaomicron* and *B. distasonis* isolates tested were reported to be resistant to imipenem. A lower breakpoint than that of the NCCLS was used in this case (6.25 μg/ml *vs* 16 μg/ml). A high-level resistance to imipenem was found to be rare among *B. fragilis* isolates collected in Nottingham (118). However, *B. fragilis* isolates which exhibited a reduced susceptibility to imipenem, with MICs of 2-4 μg/ml, *i.e.* concentrations up to 50 times higher than those for "normal" sensitive strains, were more common, accounting for 7% of the *Bacteroides* isolates from clinical specimens. In the present study, 7 (2.91%) of the 242 isolates of *Bacteroides* spp. obtained from different parts of Hungary during 1998-2000 possessed the *cfiA* gene, which is substantially more than reported from France (2.4%), but similar to the rate observed by Edwards *et al.* None of the isolates belonging in non-*fragilis* species of *Bacteroides* gave positivity in the PCR experiments. 5 of the *cfiA*-positive strains would be classified as not resistant by the conventional breakpoint criterion (MIC ≥16 μg/ml). However, for 17% of the *B. fragilis* and

64% of the non-*fragilis* isolates, the MIC values were at least 10 times higher than those for the fully-sensitive strains.

It has been shown that specific events are needed for the expression of such resistances; IS elements carrying outward-oriented promoters should be inserted upstream of the 'silent' resistance genes, and the prevalence of these 'silent' genes and the activating IS elements in the population should be "high" enough. These isolates have the potential to convert to a high level of metallo-β-lactamase expression and resistance in consequence of the presence of an insertion element carring an efficient promoter immediately upstream of the *cfiA* gene. The frequency of 'silent' and phenotypically imipenem-resistant *Bacteroides* isolates seems to be low in countries where the prevalence of the *cfiA* gene has been examined. In Hungary, the prevalence of 'silent' *cfiA*-positive and real imipenem-resistant isolates was 2.0% and 0.8% respectively, according to the data of this study. These figures are similar to or slightly lower than the values reported in other countries.

IS elements were found upstream of the *cfiA* gene in our carbapenem-resistant strains: IS*942* was confirmed in the right position upstream of the gene, which explains the higher MICs found in this case.

The 729 bp PCR product of the strain *B. fragilis* isolate 22 escaped detection by hybridization with high stringency washes, because of its lower homology to the probe from TAL3636. However, nucleotide sequencing revealed that this fragment is *ca* 96% homologous to the described *Bacteroides cfiA* genes, and it therefore really does represent a carbapenemase gene fragment. The phenomenon of the *cfiA* probe not hybridizing with all carbapenemase genes was reported by Ruimy *et al.*, when DNA of *B. fragilis* strains belonging in homology group II did not show homology to a *cfiA* internal fragment (119).

At present, few *Bacteroides* strains have been reported to harbour the *cfiA* gene and/or express metallo-β-lactamase production resulting in carbapenem resistance to the strains and causing a possible failure of therapy. The presence of the *cfiA* gene in *B. fragilis* isolates from faecal flora and in faecal samples was likewise confirmed by Fang *et al.*, and other species than *B. fragilis* have also been isolated with a high-level production of metallo-β-lactamase. Careful following of the changes in the imipenem resistance of *Bacteroides* strains isolated from clinical samples and from normal faecal flora may be important, together with regular screening for the *cfiA* gene in these isolates. The high numbers of *Bacteroides* isolates which exhibit a reduced susceptibility to imipenem that have been reported in different studies, including ours, may draw attention to a probable decrease in the resistance breakpoint of imipenem.

5.5. 16S rDNA PCR-RFLP analysis of *Bacteroides* spp. to improve species identification

The members of the *Bacteroides* spp. are isolated most frequently from human clinical material, but routine phenotypic identification can be laborious and is often unsuccessful with commercial kits. The variable biochemical activity of strains in species and the dependence of cellular physiological responses upon the precise media and environmental conditions offers considerable opportunity for inaccurate identification and poor reproducibility. A discriminatory, reproducible and practical method for the characterization of clinical isolates may help elucidate the occurrence ad significance of these strains.

Amplified rDNA restriction analysis (ARDRA) has been used to identify various fungi and bacteria, and this method has proved to be useful for the discrimination of various anaerobic bacterial species (*Actinomyces, Propionobacteria, Lactobacillus* and *Bifidobacteria)*. The genus *Bacteroides sensu stricto* has undergone dynamic taxonomic changes in recent years and at present contains 12 species of anaerobic non-spore-forming, Gram-negative bacilli. In this study, ARDRA was applied to reference strains and clinical isolates of *Bacteroides* spp. Clinical isolates were identified initially at the species level by conventional biochemical tests and comparison of the results with those for reference strains.

In the present study, the types generated by the combination of *Taq*I and *Hpa*II endonuclease digestion profiles correlated well with the findings obtained by conventional biochemical methods and the API 20A method, and allowed the identification of the clinical strains at the species level. Initial phenotypic identification was accurate for only 70% of the isolates. When species were represented by single reference strains, all strains gave distinct profiles, and none was misidentified. The 16S rDNA PCR-RFLP approach offered an alternative to conventional methods, permitting the accurate grouping of strains and identification of *Bacteroides* spp. strains at species level. In conclusion, this method has been shown to be a simple, rapid, cost-effective, and highly discriminatory method for the identification of *Bacteroides* spp. of clinical origin.

6. CONCLUSIONS

6.1. For the first time in Hungary, we have investigated and published the epidemiology of *C. difficile* diarrhoea in hospitalized patients. The overall *C. difficile* toxin-positivity rate was 18.8% for those who contracted diarrhoea during their stay in the University Hospital of Szeged. There was no significant difference in toxin positivity rates between patients for whom *C. difficile* toxin testing had been specifically requested and those for whom it had not. The results of this study clearly demonstrated that infectious diarrhoea in hospitalized patients is more likely to be caused by *C. difficile* than by any other enteric pathogen.

6.2. A retrospective study involving molecular characterization of case isolates of *C. difficile* was performed to delineate endemic strains in our hospital. A comparison of the ribotypes of nosocomial *C. difficile* isolates originating from different patients in Szeged, Hungary, with the library of *C. difficile* ribotypes in the ARU, UK, revealed a very different distribution of PCR ribotypes. The most predominant ribotype in our Hungarian survey of *C. difficile* isolates was PCR ribotype 087, a toxigenic type, which accounted for 39% of all isolates, in contrast with an international typing study, where its prevalence was much lower. The prevalence of this ribotype in Hungary is in contrast with the findings in some other countries, where the most common ribotype is ribotype 1. A previously unrecognized type was found in this study.

6.3. In this study, the first high-level metronidazole-resistant *Bacteroides* strain and the first intermediate-resistant *Bacteroides* strains were found and published in Hungary, harbouring the metronidazole resistance determinant, the *nim* gene. The prevalence of the *nim* resistance genes among our clinical isolates from *Bacteroides* spp. was 1.5%. We characterized the *nim* genes which were found: the *nimA* and the *nimB* gene were detected in the intermediate-resistant strains, both of them harbouring the upstream copies of the *IS1186/IS1168* element. A newly recognized *nim* gene (*nim*E) was present in our high-level metronidazole-resistant strain.

6.4. For the first time in Hungary, this study investigated the prevalence of the carbapenemase gene of the clinical isolates of *Bacteroides*. 2.91% of the *Bacteroides* spp. obtained from different parts of Hungary possessed the *cfiA* gene. The survey on the occurence of 'silent', but *cfiA*-positive, and phenotypically resistant *B. fragilis* isolates demonstrated their low prevalence (0.8%) in Hungary. These strains probably comprise a distinct subspecies of *B. fragilis*: IS*942* was confirmed in the right position upstream of the *cfiA* gene in the carbapenem-resistant strains.

6.5. We developed amplified 16S ribosomal DNA restriction analysis for identification of the species level of *Bacteroides* in Hungary. This method offered an alternative to conventional methods, permitting the accurate grouping of strains.

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Annex