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

Summary of PhD Thesis

Edit Urbán

Institute of Clinical Microbiology,
Faculty of Medicine,
University of Szeged, Hungary

2002

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.

Clostridium difficile is recognized as the major infectious agent responsible for nosocomial diarrhoea and potentially lethal pseudomembranous colitis (PMC) following antimicrobial therapy. 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. One of the most remarkable characteristics of such diseases is that, in almost all cases, they occur following antibiotic therapy. Cumulative researches pointed the finger of suspicion at toxin-producing *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*. 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. Nevertheless, during outbreaks, higher acquisition rates have been observed, such as 32% in the study performed by Delmée in a haematology ward.

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. 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*. 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. 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.

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. *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 respiratory 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). *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. 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. 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.

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. 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. 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.

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

Epidemiological investigation of *C. difficile*

Bacterial strains

During a one-year period, 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.

Toxin testing

Toxin B: Cytotoxin assay by tissue culture: Freshly-taken faecal samples were used for the standard procedure for the cytotoxin assay, using the HeLa cell line. 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.

Toxin A: was determined by using the VIDAS (bioMérieux, France) toxin detection kit, according to the manufacturer's indtruction.

PCR ribotyping of clinical isolates of *C. difficile*

Bacterial strains

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).

PCR ribotyping method

Isolates were cultured anaerobically overnight under anaerobic conditions on FAA 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, 10 µl of supernatant was added to 90 µl of PCR mix. 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.

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.

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.

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

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.

***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. 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.

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).

Detection and mapping of the activating IS elements

The presence of the *IS1186* element was determined with the primer pairs *IS1186A* and *IS1186B*. *IS1186* elements were mapped upstream of the *nimA,B* genes, using the NIM-5 and IS1186A primers. 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).

Plasmid isolation and Southern hybridization

Plasmid DNA was isolated as described before, 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, 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).

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

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.

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 48h in an anaerobic environment (Bactron, Shell Lab., Cornelius, USA).

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.

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.*

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 were used as template DNA. 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.

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.

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

Bacterial culturing and identification of strains

Bacterial strains were cultured on FAA 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. 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.

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. 16S rDNA genes were amplified with the universal primers pA and pH. Template nucleic acid (5 µl) was included in a 50 µl PCR reaction mixture 0.2 µM of each primer, and 1U of *Taq* DNA polymerase [Promega, Madison, Wisconsin, USA]).

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.

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).

4. RESULTS AD DISCUSSION

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. No isolates of other enteropathogenic bacteria (*Yersinia* sp., *Shigella* sp. or enteropathogenic *E. coli*) were found during this period.

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. 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. 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 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.

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

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. All of the isolates originated from different patients in various wards at the University Hospital of Szeged, none of them were outpatients. During a period of 3 months, 57 *C. difficile* strains were isolated from 252 faecal samples; 44 of them (77%) were toxin-producing. Six of the 8 clinical samples other than faeces collected during the same period (75%) were toxin-producing. A total of 15 different ribotypes were detected among the 65 isolates tested. 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). 2 non-toxigenic isolates belonging in ribotype 010 were isolated from conjunctiva and newborn faeces.

All but 2 of the investigated isolates could be typed by this PCR ribotyping method; the exceptions were non-toxigenic isolates. 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.

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. 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.

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

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. All of them, except one high-level metronidazole-resistant strain, were susceptible to metronidazole (NCCLS resistance breakpoint 32 μg/ml), but 4 of them (2.4%) in 2000 and 14 (7%) in 2001 exhibited reduced sensitivity. The MIC_{90} was 1.0 μg/ml in both years.

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. 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. 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. *B. fragilis* 19924, gave a metronidazole MIC of 12 μg/ml, had a PCR RFLP profile consistent with *nimA*, and the *B. fragilis* 29877/1 with a metronidazole MIC of 4 μg/ml, had a PCR RFLP profile consistent with *nimB* gene. *B. fragilis* 20384, gave a high-level metronidazole MIC (>256 μg/ml) and had a PCR RFLP profile consistent with *nimE*. In addition to the *nim* gene types, the presence of the activating IS elements was also determined. 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. 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.

Metronidazole is the first drug of choice for the empirical coverage of anaerobic infections. The first *B. fragilis* strain resistant to metronidazole was reported by Ingham *et al.*

in 1978. 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. 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. 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. 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.

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. 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. 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), 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. 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.

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.

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

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.

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. 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. 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

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.

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.

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.

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.

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.

Communications connected with the thesis

I. Sóki József, **Urbán Edit**, Szőke Ildikó, Fodor Eleonóra, Nagy Erzsébet:
Prevalence of the carbapenemase gene (*cfiA)* among clinical and normal flora isolates of *Bacteroides* in Hungary
J. Medical Microbiology, **49**:427-430 (2000) **I.F.: 1.625**

II. Nagy Erzsébet, Sóki József, Edwards Rchard, **Urbán Edit,** Lajos Zoltán, Szabó Béla:
Az első hazai carbapenemase termelésen alapuló imipenem rezisztens *Bacteroides fragilis* törzsek genetikai vizsgálata
Infektológiai és Klinikai Mikrobiológia, **7**:65-66 (2000)

III. Urbán Edit, Terhes Gabriella, Nagy Erzsébet:
Milyen gyakran kell számolni fekvőbeteg intézetben *Clostridium difficile* által okozott hasmenéssel?
Infektológiai és Klinikai Mikrobiológia, **7:**137-140 (2000)

IV. Tusnádi Anna, Bakró Ildikó, **Urbán Edit**, Nagy Erzsébet:
Clostridium difficile hasmenés két megyei kórházban.
Infektológiai és Klinikai Mikrobiológia, **7:**133-136 (2000)

V. József Sóki, Richard Edwards, **Edit Urbán**, Eleonóra Fodor, Elisabeth Nagy:
A clinical isolate of *Bacteroides fragilis* from Hungary with high-level resistance to imipenem
J. Medical Microbiology, **50**:107 (2001) **I.F.: 1.625**

VI. József Sóki, Richard Edwards, Zoltán Lajos, Tibor Vrabély, Eleonóra Fodor, **Edit Urbán,** Elisabeth Nagy:
Isolation and characterisation of an imipenem-resistant *Bacteroides fragilis* strain from a prostate abscess in a dog
Veterinary Microbiology, **84:** 187-190 (2002) **I.F.: 1.428**

VII. Elisabeth Nagy, József Sóki, **Edit Urbán**, Ildikó Szőke, Eleonóra Fodor, Richard Edwards:
Occurrence of metronidazole and imipenem resistance among *Bacteroides fragilis* group clinical isolates in Hungary
Acta Biologica Hungarica, **52:** 271-280 (2001) **I.F.: 0.291**

VIII. Edit Urbán, Jon S. Brazier, József Sóki, Elisabeth Nagy, Brian I. Duerden:
PCR-ribotyping of clinically important *Clostridium difficile* strains from Hungary
J. Medical Microbiology, **50:**1082-1086 (2001) **I.F.: 1.625**

IX. Edit Urbán, Anna Tusnádi, Gabriella Terhes, Elisabeth Nagy:
Prevalence of gastrointestinal disease caused by *Clostridium difficile* in a University Hospital in Hungary
Journal of Hospital Infection **51:**175-178 (2002) **I.F.: 1.812**

X. Edit Urbán, József Sóki, Jon S. Brazier, Elisabeth Nagy, Brian I. Duerden:
Prevalence and characterization of *nim* genes of *Bacteroides* spp. isolated in Hungary
Anaerobe (accepted for publication) **I.F.: 0.410**

Acknowledgements

I wish to express my special thanks to all those without whom this task would never have been fulfilled:

I am greatly indebted to my supervisor, **Professor Elisabeth Nagy**, whose scientific guidance, encouragement and support over the past years have been invaluable and for which I am deeply obliged.

I express my grateful thanks to **Professor Brian I. Duerden**, head of the Anaerobe Reference Unit in Cardiff, for his professionalism in his supervision of my study in Cardiff.

I am very grateful to **Dr. Jon S. Brazier** for sharing with me his vast knowledge on the ribotyping method of *C. difficile* and the PCR method of *nim* gene. Special thanks are due to him for his untiring efforts and constant enthusiasm.

I wish to thank **Dr. Richard Edwards** for his wide-ranging cooperation.

I am especially grateful to all my coworkers, **Dr. József Sóki, Dr. Eleonóra Fodor and Gabriella Terhes,** for their helpful cooperation during our work.

I am especially grateful to **Anita Bönde and all the members of the staff of the Institute of Clinical Microbiology,** whose indispensable technical assistance has contributed so much to this work.

And last, but not least I wish to thank **my family,** my husband Ernő, my daughter Gerda and my son Ádám, for the love and support I have received during these years.