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

Investigation of some clinically important toxin-producing anaerobic bacteria and their epidemiology

Ph.D Thesis

Gabriella Terhes

2006
Szeged

1. INTRODUCTION

1.1. Studies involving *C. difficile*

1.1.1. Historical overview of *C. difficile* infections

Clostridium difficile (C. difficile) is a Gram-positive obligate anaerobic spore-forming rod, which was first isolated from the meconium and faeces of a newborn baby by *Hall* and *O'Toole* in 1935; they termed their isolate *Bacillus difficilis*. In 1893, *William Osler* and *John Finney* first described pseudomembranous colitis (PMC) in a young woman. They did not determine the pathogenic microorganism in this clinical picture, but *C. difficile* is now known to be the main causative agent in PMC. In 1974, *Tedesco* reported PMC after clindamycin therapy; in spite of this, the role of this bacterium in this clinical picture was clarified only after 1978. At the end of the 1980s, 60-75% of antibiotic-associated colitis (AAC) and 11-33% of antibiotic-associated diarrhoea cases (AAD) were related to *C. difficile* as a pathogen; asymptomatic colonization is probably 2 to 5-fold more common than the disease itself. Since the 1990s, the number of diagnosed cases of *C. difficile* infection has increased, because of the development of identification and other laboratory methods. The annual reports of the WHO and our own study indicate that *C. difficile* is now the most frequent nosocomial pathogen; we presume that this tendency will continue and the presence of this bacterium in community-acquired diarrhoea is likely to increase because of the wide application and in many cases the misuse of different antibiotics. The severity and spread of this infection and the frequency of treatment may place a burden on the public health system.

1.1.2. Virulence factors of *C. difficile*

Numerous putative virulence factors of this bacterium have been identified, but in many cases their role in the pathogenesis of *C. difficile* infection is not well determined. The main virulence factors are two well-characterized exotoxins, toxin A and toxin B; a third, less well-known exotoxin is actin-specific ADP-ribosyl transferase (binary toxin). Researchers originally believed that there were two types of *C. difficile* strains: toxin-producing strains (toxin A- and toxin B-positive) and toxin-negative strains. At present, several toxin variant strains have been identified: toxin A-negative, toxin B-positive, and binary toxin-negative strains or toxin A-negative, toxin B-positive and binary toxin-positive strains, most of them with various deletions at the 3' end of the toxin A gene. Toxins A and B share numerous similar structural features, such as three main domains: the enzymatic domain with glucosyltransferase activity is located at the N-terminal region; in the middle of the toxin, there is a central hydrophobic region; and the C-terminal region is responsible for recognizing the target cell receptor and the binding to the host cell. Toxins A and B have monoglucosyltransferase activity, which catalyses the incorporation of glucose into Thr-37 of RhoA. The modified Rho is unable to maintain the normal organization of the target cell cytoskeleton, resulting in typical cell rounding.

The third, less well-known toxin is an actin-specific ADP-ribosyltransferase, which consists of two subunits, CDTa and CDTb. CDTa is an enzymatic component, while CDTb is responsible for the binding of this toxin to the target cells and contributes to the translocation of the CDTa subunit into the host cells. Both subunits are encoded by separate genes, *cdt*A and *cdt*B. In the target cell, the enzymatic component of the binary

toxin translocates from the endocytotic vesicle into the cytoplasm, where CDTa catalyses the ADP-ribosylation of the cytoskeletal actin, causing actin disorganization.

1.1.3. Risk factors in *C. difficile* infections

After the description of *C. difficile* as a nosocomial pathogen, numerous risk and host-related factors were determined; these may contribute to the development of *C. difficile* infection, for example acquisition of this microorganism and its toxin production; disruption of the normal intestinal flora by antibiotic therapy or prophylaxis and chemotherapy; long-term hospitalization; age-related susceptibility (mainly $\geq$ 65 years); severe underlying disease and intensive care or other surgical procedures. During *C. difficile* infection, the symptoms range from asymptomatic colonization through mild diarrhoea to PMC or toxic megacolon, colonic perforation and rarely death.

1.1.4. Typing of *C. difficile* strains

In consequence of the emergence of new toxin variant strains and the spread of toxin A/B-positive or toxin A-negative, and toxin B-positive strains in hospitals and the community, culturing methods and further investigations of these strains may be important for elucidation of the exact role of toxin variant strains in the pathogenesis and for control of their spread. Numerous phenotypic and genotypic methods have been used internationally to study *C. difficile* strains of different origins, such as resistance pattern, bacteriocin and bacteriophage typing methods, AP-PCR, RFLP, PFGE and PCR ribotyping. PCR ribotyping, the method most widely used to determine the intraspecies genetic variation of *C. difficile*, in which the 16S and 23S rRNA intergenic spacer region can be amplified by using specific primers under stringent amplification conditions. The sizes of the amplification products can range from 250 bp to 600 bp.

1.2. Studies involving *B. fragilis*

1.2.1. Historical overview of *B. fragilis* infection

Bacteroides fragilis (*B. fragilis*), a Gram-negative anaerobic microorganism, constitutes about 1-2% of the normal colonic bacterial flora in humans. However, it has also frequently been isolated from intraabdominal abscesses, soft-tissue infections, diabetic ulcers and sepsis. In 1984, *Myers et al.* first described *B. fragilis*-associated diarrhoea in lambs. Further investigations demonstrated that *B. fragilis* strains can cause diarrhoeal diseases in piglets, foals and calves. *Myers* later observed the presence of enterotoxigenic *B. fragilis* (ETBF) strains in human diarrhoea. ETBF strains are mainly associated with acute childhood (under 5 years) diarrhoea, the majority of healthy adults carrying these strains without symptoms.

1.2.2. Virulence factors of *B. fragilis*

B. fragilis possesses numerous virulence factors, which can cause damage in the human host under certain circumstances, such as abdominal infections and abscess formation. The main virulence factors are enterotoxin production, capsule, LPS, special surface proteins, an iron uptake mechanism and the presence of various resistance genes. The *B. fragilis* enterotoxin (fragilysin or BFT) is a 20.7 kDa single-chain protein, a Zn-dependent metalloprotease belonging in the metzicins family. The *bft* gene is located within the 6 kb pathogenecity islet of the ETBF strains, which is similar to other pathogenecity islands in pathogenic *E. coli* or *Salmonella* strains. In non-toxigenic

(NTBF) strains, the *bft* gene and the island are not present. At present, there are three different allelic forms of the toxin-coding gene, designated *bft*-1, *bft*-2 and *bft*-3. *In vitro* and *in vivo*, this toxin can damage the ileal and colonic mucosa in various animals and humans. During the toxic effect, the intestinal permeability and chloride secretion may increase. Moreover, this toxin can cause a reversible cytotoxic effect on the HT-29 cell line.

Since the 1960s, the widespread use of various antibiotics in anaerobic infections has resulted in the selection of resistant *B. fragilis* strains. Up to this point, only a limited number of antimicrobial agents have been available for the treatment of anaerobic infections caused by *B. fragilis* strains, such as piperacillin/tazobactam, imipenem, cefoxitine, chloramphenicol and metronidazole; additionally, multiresistant strains have been observed, though their incidence is low. Imipenem-resistant strains, with a prevalence of about 0.7-3%, can produce a metallo-β-lactamase coded by the *cfi*A gene. The *cfi*A gene can exhibit various expression levels: it may be silent or in some cases the strains can show extended resistance to β-lactams and β-lactamases.

1.2.3. The role of enterotoxigenic *B. fragilis* in different infections and possibilities for its diagnosis

B. fragilis is a member of the normal colonic microflora, as mentioned above, and at the same time the anaerobic microorganism most frequently isolated from various human infections. ETBF strains may cause severe diarrhoea among farm animals, and a correlation has been demonstrated between the isolation of ETBF strains and diarrhoea in 1-5-year-old children; the presence of these strains in the bowel does not always lead to intestinal mucosa damage, mainly among adults, where the asymptomatic colonization is particularly high. Many researchers have shown that the *bft* gene is more prevalent among strains from blood cultures; the frequency varies between 26 and 52%, and an increased frequency of fragilysin production has also been detected in extraintestinal isolates (14-21%).

It is well known that *B. fragilis* is a significant opportunistic pathogen, its rapid identification is therefore important for clinical microbiology. Culturing, biochemical tests and antibiotic susceptibility tests are time-consuming methods, and thus alternative identification techniques are necessary for accurate and rapid diagnosis; the use of rRNA-RFLP may help solve this problem. The diagnostic tools for the detection of ETBF strains are limited to some methods: cytotoxicity assays on different cell lines, and molecular methods. At present, no commercial diagnostic kits for this purpose have been developed. Most routine laboratories apply only culturing and biochemical methods or sometimes molecular methods (PCR-RFLP) for the identification of *B. fragilis*, and merely some of them use cell toxicity assays for the detection of toxin production, though this method is important to distinguish NTBF and ETBF strains from childhood diarrhoeal cases.

1.2.4. Typing of *B. fragilis* strains

In the last ten years, numerous molecular typing methods, such as rRNA-RFLP, REA (restriction endonuclease analysis), ribotyping, AP-PCR and PFGE have been used to study the polymorphism of *B. fragilis*. Most of these typing studies revealed that there are at least two genetically distinct groups of *B. fragilis* strains, one group carrying the *cfi*A gene, which encodes a metallo-β-lactamase (Amber's class B), while

the second group is generally *cep*A- (Amber's class A β-lactamase) or *bft*-positive. The *cfi*A-positive group exhibits a lower recombination rate, while a high level of housekeeping gene recombination can be observed in *cep*A- or *bft*-positive strains.

2. AIMS OF THIS STUDY

In Hungary, the presence of binary toxin-producing *C. difficile* strains has not been analysed so far. Accordingly, we set out to study the presence of

1. the *tcd*A and *tcd*B genes, which encode the major toxins: toxins A and B;
2. *cdt*A and *cdt*B, which are responsible for binary toxin production;
3. deletion at the 3' end of toxin A gene; and
4. the CPE of *C. difficile* strains originating from faecal specimens on the HeLa cell line.

Earlier (2001), in cooperation between the Anaerobe Reference Unit (ARU, Cardiff, UK) and the University Hospital of Szeged (Hungary), 65 *C. difficile* isolates were typed by using a PCR ribotyping method. The aims of the present study were

5. to extend this investigation and to determine the distribution of PCR ribotypes in three Hungarian regions;
6. to compare the results with earlier ribotyping study findings; and
7. to analyse the results according to the patient group (inpatients and outpatients).

In Hungary, the incidence of ETBF strains in local clinical samples was earlier studied by using cell cytotoxicity assays. We now planned

8. to investigate the presence of the *bft* gene among Hungarian *B. fragilis* strains originating from various clinical specimens;
9. to determine the main *bft* types among *bft*-positive isolates;
10. to study the coincidence of the *bft* and *cfi*A genes in different strains;
11. to analyse the first *bft*-positive and *cfi*A-positive *B. fragilis* strain isolated from severe infection in the UK; and
12. to study *bft* or *cfi*A-positive isolates by using an ERIC PCR typing method.

3. MATERIALS AND METHODS

3.1. *C. difficile* strains

118 isolates of *C. difficile* were examined to detect the main toxin genes, *tcd*A, *tcd*B, *cdt*A and *cdt*B, and the variation at the 3' end of the *tcd*A from faecal specimens of both outpatients and inpatients. These strains were isolated in Hungarian laboratories in the towns of Szeged, Szolnok (South Hungary), Budapest (Budapest region) and Veszprém (West Hungary) between 2002 and 2004. 105 of 118 strains were used to determine the main ribotypes in the different Hungarian regions; 65 of the 105 isolates were obtained from inpatients, and 40 from outpatients. The reference strain VPI 10463 (toxinotype 0) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Binary toxin-producing control strains R5989 (ribotype 023), R8637 (ribotype 019) and R10456 (ribotype 058) were supplied by Jon Brazier (ARU, Cardiff, UK). The strains were grown on anaerobic blood agar plates and in brain-heart infusion broth. The cultures were incubated in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2, Bactron, Sheldon Men. Inc., USA) for 24 h at 37 °C in the case of toxin gene PCR, while

for ribotyping, the examined strains were grown on Fastidious Anaerobe Agar (LabM, Bury, UK); the plates were incubated at 37 °C for 24 h under anaerobic conditions.

3.2. *B. fragilis* strains

275 *B. fragilis* strains were isolated from different clinical specimens in 5 laboratories (Szeged, Székesfehérvár, Budapest, Debrecen, and Kiskunhalas) in Hungary, and one *B. fragilis* strain R19811 was obtained from the ARU, Cardiff strain collection to examine the presence of the *bft* and *cfi*A genes. This strain was isolated from a blood culture in the UK last year. The strains were grown on anaerobic blood agar plates and in brain-heart infusion broth. The cultures were incubated in an anaerobic cabinet for 24-48 h at 37 °C. The strains were identified by conventional methods, ATB rapid ID 32A (BioMerieux, France) and rRNA-RFLP in the case of the *B. fragilis* isolate from the ARU strain collection.

3.3. Toxin assays for detection of *C. difficile* and *B. fragilis* toxins

The pure overnight broth cultures (*C. difficile* or *B. fragilis*) were centrifuged at 12 000 rpm for 10 min, the supernatants were filtered through a 0.22 μm pore-size membrane (MILLEX®-GV), and the filtrate was frozen immediately and kept at –20 °C until used. HeLa cells for the detection of *C. difficile* toxins A and B were grown at 37 °C in Dulbecco's modified EAGLE's medium (Sigma, St. Louis, USA) supplemented with 10% heat-inactivated foetal bovine serum, while the HT-29 cell line, which is suitable for the detection of *B. fragilis* fragilysin, was grown in McCoy`s 5A medium with glutamine (Sigma, St. Louis, USA) supplemented with 10% heat-inactivated foetal bovine serum at 37 °C. Before the cytotoxicity assay, the cell culture medium was removed and fresh medium was added without serum. Ten-fold dilutions of filtered bacterial culture supernatants were inoculated into the microtitre wells containing semi-confluent HeLa or HT-29 cells and, after incubation at 37 °C, in 5% CO_2, for 24 h in the case of *C. difficile*, the CPE was examined. The CPE of ETBF was detected after exposure for 1, 2 and 4 h at 37 °C, in 5% CO_2. The observed CPEs of different strains were compared with those of the positive control strain (VPI 10463 *C. difficile* and BF-379 *B. fragilis*) and the negative control (PBS). *C. difficile* goat antitoxin serum was used for the toxin-neutralization test; this antitoxin serum neutralizes both toxins A and B.

3.4. Detection of *C. difficile* toxin genes by PCR assays

A single colony of *C. difficile* was suspended in TE buffer (50 mM Tris hydrochloride pH 8.0, 5 mM EDTA and 50 mM NaCl), and the suspension was heated at 95 °C for 10 min, and then centrifuged at 12 000 rpm for 5 min. NK2 and NK3 primers were derived from the non-repeating sequence of the toxin A gene, while NK104 and NK105 were from the sequence of the toxin B gene. NK9 and NK11 were from the repeating sequence of the toxin A gene; these primers were used to detect the deletion or insertion of the 3' end of the toxin A gene. The reaction mixture (20 μl) contained 1 μl of template DNA, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 μg/ml gelatine, 0.2 mM dNTP, 0.3 U *Taq* DNA polymerase supplied in 10 μl Ready Mix (Sigma, St. Louis, USA) and 0.15 μM cdtApos, cdtArev, cdtBpos and cdtBrev (*cdt*A and *cdt*B PCR), or 45 ng of the NK2, NK3, NK9, NK11, NK104 and NK105 primers. The reaction mixtures were incubated for 30 (*cdt*A and *cdt*B PCR) or 35 cycles (*tcd*A and *tcd*B PCR) in a PCR system (GeneAmp, PCR system 9600, Perkin Elmer, Norwalk, CT,

USA). Gene amplification consisted of denaturation at 94 °C for 45 s (*cdt*A and *cdt*B) or at 95 °C for 20 s (*tcd*A and *tcd*B), annealing at 52 °C for 60 s (*cdt*A and *cdt*B) or at 62 °C for 120 s (*tcd*A and *tcd*B), and extension at 72 °C for 120 s (*cdt*A and *cdt*B). PCR products were visualized by running in 0.8-1.5% agarose gels with ethidium bromide (0.5 µg/ml) at 5 V/cm in Tris-borate EDTA buffer (45 mM Tris-borate, 1 mM EDTA). Gels were photographed under UV light with a Kodak digital camera and analysed with Kodak 1D 3.5 software.

3.5. Detection of *C. difficile* binary toxin by Western blotting and toxinotyping

These assays were performed by Frederic Barbut (Laboratoire de Bactériologie, Faculté de Médecine, UFR Saint-Antoine, Université Paris).

3.6. Detection of fragilysin gene (*bft*) and determination of *bft* isoforms by PCR

One colony of pure, overnight cultures was suspended in 50 µl TE buffer, and then incubated at 95 °C for 10 min. The suspensions were centrifuged at 12 000 rpm for 5 min. The supernatants were transferred to sterile Eppendorf tubes, frozen immediately and kept at -20 °C until used. The *bft* and *bft* typing PCR were performed in a PCR system. The reaction volume for the *bft* PCR was 25 µl, while for the *bft* typing it was 50 µl. The regular reaction mixture (see in section 3.4) contained 10 pmol BF1 and BF2 primers (*bft* PCR), and 70 pmol BTT1 and BTT2 modified primers (*bft* typing), 1 µl (*bft* PCR) or 5 µl (*bft* typing) of cell lysate supernatant and 1.25 U *Taq* polymerase (Sigma, St. Louis, USA). The PCR profile was 35 cycles, 1 cycle consisting of denaturation at 94 °C for 1 min (*bft* PCR) or at 94 °C for 30 s (*bft* typing), annealing at 52 °C for 1 min (*bft* PCR), or at 54 °C for 1 min (*bft* typing), and extension at 72 °C for 1 min (*bft* PCR) or at 72 °C for 3 min (*bft* typing). The *bft* typing PCR products were purified by using an ethanol precipitation method and then digested with the restriction enzyme *Mbo*I (Fermentas, Vilnius, Litvania) according to the manufacturer`s recommendation. This enzyme has different recognition sites in the isoforms *bft*-1, *bft*-2 and *bft*-3; the restriction digestion therefore gives products of different lengths. PCR products were visualized by running in 1.5% agarose gel, while the digestion products (*bft* typing) were separated in 1.5% Metaphore agarose (Cambrex Bio Science, Rockland, ME, USA).

3.7. PCR ribotyping of *C. difficile* strains

After checking the purity of the culture, the cells were resuspended in a 5% (wt/vol) solution of Chelex-100 (Bio-Rad, Hemel Hempstead, UK). The solutions were incubated in boiling water for 12 min and then centrifuged at 12 000 rpm for 10 min. The supernatant was removed to a fresh tube, and stored at 4 °C for 12-24 h. Amplification reactions were performed with a 100 µl volume containing 50 pmol of each primer (16 S rRNA gene primer **p3** 5'-CTG GGG TGA AGT CGT AAC AAG G-3'; position 1445-1466, and 23S rRNA gene primer **p5** 5'-GCG CCC TTT GTA GCT TGA CC-3'; position 1-20), 200 µM each deoxynucleoside triphosphate (Pharmacia), 10 mM Tris/HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (Pharmacia) and 10 µl template DNA. The PCR was carried out in a thermal cycler, for 1 cycle of 120 s at 95 °C for starting denaturation; 30 cycles of denaturation at 92 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s. After the last denaturation step, annealing was performed at 55 °C for 45 s, and extension at 72 °C for 5 min. This method was developed previously in the ARU (Cardiff, UK). Amplification products

were concentrated to a final volume of 25-30 µl in the heat-block set at 75 °C for 1.5 h. PCR products were fractionated by electrophoresis through 3% (w/v) Metaphore agarose (BMA-BioWhittaker Molecular Applications, Rockland, ME, USA) for 3 h, at 200 V, 60 mA in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer. The pattern was visualized after staining for 20 min in ethidium bromide (0.5 µg/ml) under UV light. Ladder (Superladder-Low, ABgene, UK) should be spaced every 6 wells to enable normalization of the gel patterns. PCR ribotype profiles were analysed with GelCompar image analysis software (version 4.0; Applied Maths, Kortrijk, Belgium).

3.8. Extraction of outer membrane-, and S-layer protein of *C. difficile* and their detection by SDS-PAGE

The log-phase starter culture was prepared in prereduced CMB (cooked meat bouillon) at 37 °C for 24 h. After checking the purity of the culture, this was used to inoculate the selected test medium (prereduced peptone-yeast extract), which was incubated at 37 °C for 24 h. 20 ml of log-phase broth culture was centrifuged at 5 000 rpm for 20 min at 24 °C. Harvested cells were washed in cell washing buffer (0.05 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 0.15 M NaCl). The suspended cells were centrifuged again at 5 000 rpm for 20 min at 24 °C, and suspended in 1 ml of EDTA buffer (0.05 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 0.15 M NaCl, 0.01 M EDTA) or 1.125 ml of 5 M guanidine hydrochloride by vortex mixing, and the cells were transferred to a sterile tube. The guanidine hydrochloride protein extract was incubated at 24 °C for 2 h with shaking. After the incubation period, the cells were centrifuged twice at 13 000 rpm for 2 min, and the cell-free supernatant was transferred to a sterile Eppendorf tube and stored -20 °C until the dialysis was performed. The tube with the EDTA extract was placed into a 45 °C waterbath for 30 min, after which the cells were mixed for 10 s, sonicated in a sonic bath for 1 min, and then mixed again for 10 s. The sonicated cells were transferred to a sterile Eppendorf tube, and centrifuged at 12 000 rpm for 2 min. The supernatant with EDTA was centrifuged again to ensure the removal of bacterial cells, and the EDTA was removed from the cell-free supernatant by dialysis (dialysis buffer 0.01 M Tris/HCl, 0.01% 2-mercaptoethanol) at 4 °C for 5 h. The EDTA and guanidine hydrochloride protein extracts were mixed with an equal amount of double strength SDS-PAGE buffer (0.125 M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue), and the mixture was heated in a 100 °C waterbath for 3 min. All extracts were detected on 10% separating gel and 4% stacking gel, using the buffer system of *Laemmli*. The initial voltage was 60 V until the protein reached the lower edge of the stacking gel, and then the voltage was increased to 150 V and after 4 h the separated proteins were stained with Coomassie blue.

3.9. ERIC PCR for typing of *B. fragilis* strains

After checking the purity of the culture, the cells were resuspended in a 5% (wt/vol) solution of Chelex-100 (Bio-Rad, Hemel Hempstead, UK). The solutions were incubated in boiling water for 12 min and then centrifuged at 12 000 rpm for 10 min. The supernatant was removed to a fresh tube, and stored at -20 °C. Regular PCR reactions (see in section 3.4) were performed in a total volume of 50 µl containing 5 µl of the DNA extract, 80 pmol ERIC1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG GAA GTG ACT GGG GTG AGC G-3') and 1.25 U *Taq* polymerase (Sigma, St. Louis, USA). A 35-cycle PCR with the following profile was

performed in a thermal cycler: 94 °C for 1 min, 52 °C for 1 min, 65 °C for 2 min. The starting denaturation was carried out at 94 °C for 5 min, and the final extension at 65 °C for 15 min. PCR products were detected by running in 1.5% agarose gel as described previously (see section 3.6).

3.10. Detection of the *cfi*A gene and its upstream region by PCR

The regular amplification reaction (see in section 3.4) was performed in 25 µl containing 1 µl of DNA template (see DNA template preparation in section 3.6), 17.5 pmol cfiA-1 (5'-TCC ATG CTT TTC CCT GTC GCA GTT AT-3'), and cfiA-2 primers (5'-GGG CTA TGG CTT TGA AGT GC-3'), 0.7 µM of Up2 and G primers (Table 3) to detect IS elements in the upstream region of the *cfi*A gene, and 1.25 U *Taq* polymerase (Sigma, St. Louis, USA). Amplifications were carried out in a thermocycler programmed for 35 cycles of 5 min at 94 °C (starting denaturation), 30 s at 94 °C, 1 min at 50 °C, 1 min 72 °C, followed by a final extension step of 10 min at 72 °C, in the case of *cfi*A upstream PCR, denaturation for 30 s at 94 °C, annealing for 1 min at 52 °C, and extension for 3 min at 72 °C. A 729 bp fragment of the *cfi*A gene and the product of the upstream region (1300-1800 bp) were detected in 0.7% agarose gel.

3.11. IS, *nim* and *cep*A PCR of *B. fragilis* strain R19811

The regular PCR mixture (see in section 3.4) contained 0.7 µM of the following primers: IS614-1i, and IS614-2i (unpublished primers); IS942A and IS942B; IS1169/1 and IS1169/2; IS1170/1 and IS1170/2; IS1186A and IS1186B; IS4351C and IS4351D; 1 µM of nim-3 and nim-5 primers to detect the nitroimidazole resistance gene, and 0.5 µM of cepA-1 and cepA-2 primers to detect the endogenous class A β-lactamase gene, and 1µl of template DNA (see template preparation in section 3.6). During the amplification procedure, 35 cycles were used with starting denaturation at 94 °C for 5 min and final extension at 72 °C for 10 min; every cycle included denaturation at 94 °C for 30, annealing at 48 °C for 1 min, and extension at 72 °C for 1 min (steps for IS element PCR); in the case of *cfi*A upstream PCR, denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min, while *cep*A PCR consisted of 35 cycles with denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. PCR products were detected by running in 0.5-1.5% agarose gel.

3.12. Plasmid purification from *B. fragilis* strain R19811

3 ml of pure, overnight broth culture was centrifuged at 12 000 rpm for 5 min. The cells were then resuspended in 200 µl of TGE buffer (50 mM glucose, 25 mM Tris/HCl, 10 mM EDTA, pH 8.0). 400 µl of lysis buffer (0.2 M NaOH, 1% SDS) was added to the resuspended cells, and mixing was followed by incubation on ice for 5 min. After incubation, 300 µl of ice-cold III buffer (3 M K-acetate, pH 4.9) was added and the tubes were incubated on ice for 5 min again. Precipitates were centrifuged at 12 000 rpm for 12 min at 4 °C. About 700 µl of supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v). After vortexing briefly, tubes were centrifuged at 12 000 rpm for 6 min at 24 °C. Plasmid DNA was precipitated with an equal volume of isopropyl alcohol, then centrifuged at 12 000 rpm for 12 min at 4 °C. The DNA pellet was washed with ice-cold 70% ethanol and dried at 24 °C for at least 30 min. The plasmid DNA was dissolved in 40 µl of TE buffer (10 mM Tris/HCl, 1 mM

EDTA, pH 8.0) supplemented with 0.1 μg/ml RN-ase A. Tubes were incubated at 37 °C for 30 min to eliminate the residual RNA from the sample, and the purified plasmid DNA was stored at -20 °C. The plasmid DNA was detected in 0.8% agarose gel with ethidium bromide (0.5 μg/ml) at 5 V/cm in Tris-borate EDTA buffer (45 mM Tris-borate, 2 mM EDTA, pH 8.5). Gels were photographed under UV light with a Kodak digital camera and analysed with Kodak 1D 3.5 software.

3.13. Restriction analysis of plasmid DNA and Southern blot analysis

The plasmid DNA was digested by using *Hind*III (Pharmacia Biotech) restriction enzyme according to the manufacturer`s instruction. 10 μl of the digested products, 10 μl of non-digested plasmid DNA (R19811) and pJST241 *cfi*A-positive control, IS*614* positive control, *E. coli* V571 marker, and *Hind*III marker were separated on 0.8% agarose gel, and transferred to nylon membranes (Amersham, Life Science, UK). Fluorescence labelling of the probe was carried out with the Gene Image random prime labelling kit (Amersham Life Science, UK) on the basis of the manufacturer`s recommendation.

4. RESULTS

4.1. Studies involving *C. difficile*

4.1.1. Detection of toxins A and B, and binary toxin genes by PCR; comparison of data obtained with the cell cytotoxicity assay

118 *C. difficile* strains were examined; the PCR products of the toxin A and B genes were observed in 85 strains and CPEs were demonstrated on the HeLa cell line. 33 isolates were negative for both the toxin A and B genes by PCR; none of those 33 isolates caused morphologic damage to HeLa cells either. Toxin-positive isolates had no deletions or insertions in the repeating 3' end of the toxin A gene. Three isolates were detected via the expected 375-bp (*cdt*A) and 510-bp (*cdt*B) DNA fragments by PCR with binary toxin-specific primer pairs; at the same time, both the *tcd*A and *tcd*B genes were amplified. Because of the presence of toxins A and B, which covered the CPE of the binary toxin in the cytotoxicity assay, elimination of the two major toxins was necessary in order to verify the presence of this toxin. On trypsinization of the culture supernatant, trypsin should proteolyse toxins A and B and activates the binary toxin, but this proved unsuccessful. Western blotting was therefore performed to determine the presence of CDTa and CDTb. In all cases, the presence of the binding and enzymatic components of the binary toxin was justified. On analysis of the 3' end of the *tcd*A gene in these strains, no deletion was detected with the NK9 and NK11 primer pair. The usual CPEs were observed in the cell cytotoxicity assay after a 24-h incubation. One binary toxin-positive strain was identified as toxinotype III, while two strains belonged in toxinotype IV on RFLP analysis of the PaLoc region. International studies indicate that the presence of the major toxin genes and binary toxin gene is characteristic in these types. Binary toxin-positive, toxin A-and toxin B-negative strains were not found in this study. We collected data on the patients in whom the binary toxin-positive strains were isolated. The first binary toxin-producing strain was isolated from the diarrhoeal faeces of an 8-year-old boy. He had follicular tonsillitis and therefore received amoxicillin/clavulanic acid orally. He developed severe diarrhoeal disease during the therapy, and the family doctor therefore changed the antibiotic therapy to amoxicillin, but the diarrhoea persisted. The boy was admitted to the gastroenterology unit of the Children's Hospital in Budapest.

Diarrhoeal faeces collected on the first day of his hospitalization was positive for anaerobic, Gram-positive spore-forming rods; the colonies were irregular, with a rhizoid edge, and were identified as *C. difficile* by the RapID 32 A test (BioMérieux, Marcy l'Étoile, France). Toxin A was detected by the toxin A test (Oxoid). The boy received oral metronidazole and the alternative treatment was oral *Lactobacillus* therapy. Confirmed relapses were not observed after treatment. The second binary toxin-producing strain originated from the diarrhoeal faeces of a 42-year-old woman, in whom the presumed diagnosis was infectious enteritis. She had been treated with oral sulfamethoxazole-trimethoprim for an upper respiratory tract infection, and diarrhoea developed during the antibiotic therapy. From her diarrhoeal faeces, Gram-positive, spore-forming rods were isolated by using CCFA medium, and were identified as *C. difficile*, but at that time no toxin test was performed in the local laboratory. This isolate was sent to the reference laboratory (Szeged, Hungary) for toxin detection: the presence of *tcd*A, *tcd*B, *cdt*A and *cdt*B was detected by PCR. The CPE was tested on the HeLa cell line. After the termination of antibiotic treatment, the condition of the patient improved without specific therapy against *C. difficile*, and no relapse was observed. No prior hospitalization featured in this case history either. The third binary toxin-positive strain was isolated from the diarrhoeal faeces of a hospitalized patient, who received chemotherapy because of non-Hodgkin lymphoma.

4.1.2. Results of *C. difficile* ribotyping by PCR

Of the 105 *C. difficile* isolates originating from diarrhoeal faeces, 83 carried the *tcd*A and *tcd*B genes, while 22 were *tcd*A/B-negative. No toxin A-negative and toxin B-positive isolates were observed. Twenty-three different ribotypes were detected, and a comparison with the patterns recorded in the *C. difficile* library of PCR ribotypes in the Cardiff type culture collection revealed 5 new PCR ribotypes (161, 162, 164, 165 and 166). 3 strains isolated from outpatients were new PCR ribotypes (162, 164 and 165), while 4 strains (3 strains from South Hungary, and 1 from the Budapest region) from inpatients also showed a new pattern. During the examined period, the most prevalent types among the Hungarian isolates overall were PCR ribotypes 014 (24.8%), 002 (13.3%), and 085 (8.6%). Most of the toxin-negative strains were of PCR ribotype 085 (9/22 isolates). The distribution of the ribotypes differed in the three geographical regions: in the Budapest region, the dominant types were ribotypes 014, 002 and 018 (29%, 19.4% and 12.9%, respectively), whereas 014 (28.9%), 049 (11.1%), 085 (8.9%) and 002 (8.9%) were most frequent in West Hungary, and ribotypes 012 (20.7%), 002 (13.8%) and 014 (13.8%) were characteristic in South Hungary. Type 049 (11.1%) was present only in West Hungary, ribotype 005 (9.7%) only in the Budapest region, and ribotype 035 (6.9%) only in South Hungary. The presence of binary toxin-producing strains was demonstrated in West Hungary and the Budapest region, whereas binary toxin-positive strains have not yet been detected in South Hungary. The frequencies of the various ribotypes were compared between inpatients and outpatient. When Fisher's 2-sided exact test was used, the difference was only significant in one case (ribotype 012), which was more prevalent among isolates from inpatients. In comparison with other typing methods, such as S-layer typing, all of the examined strains were typable with PCR ribotyping. This method was sufficiently discriminative; the results were reproducible, and easily interpretable. The surface layer protein variability is useful as a phenotypic marker for epidemiological investigation. This method provided a possibility

to distinguish between various strains, but the protein purification and detection of this bacterial component are time-consuming, and analysis of the protein profiles needs special software; the application of this method is therefore limited to local outbreaks.

4.2. Studies involving *B. fragilis*

4.2.1. Detection of ETBF strains in various clinical samples by using PCR and cell cytotoxicity assay; examination of the coexistence of the *bft* and *cfi*A genes

275 *B. fragilis* strains were obtained from various clinical samples, diarrhoeal faeces and normal faecal flora of healthy adults from 5 Hungarian laboratories and 1 strain from the Cardiff strain collection in order to determine the frequency of the *bft* gene, to detect the most frequent isoform of the *bft* alleles and to examine the coexistence of the *bft* and *cfi*A genes. 8 of the 275 (3%) *B. fragilis* isolates and *B. fragilis* (R19811) from the Cardiff collection were from blood cultures, 54 (20%) were from abdominal abscesses, 153 (55%) were from other infection sites, such as diabetic foot ulcers, pelvic abscesses, or BV (bacterial vaginosis), 22 (8%) were from faeces of healthy subjects and 38 (14%) were from diarrhoeal faeces.

24 of the 275 isolates and strain R19811 were *bft*-positive by the PCR method, while in 24 cases, a reversible CPE was observed on the HT-29 cell line. The weak toxin production or the sensitivity of the cytotoxicity assay may be responsible for the difference between these two methods. A majority of the examined strains (17 isolates) carried the *bft*-1 allele, while 7 showed the characteristic *bft*-2 pattern on PCR-RFLP. 14 of the 275 *B. fragilis* strains and strain R19811 carried the *cfi*A gene. In detail, 1 of the 8 (12.5%) Hungarian blood culture isolates harboured *bft* gene, the *cfi*A gene could not be detected among the examined strains. In the *bft*-positive strain from a blood culture, the *bft*-2 allele was detected by PCR-RFLP. In 2 (3.7%) strains from abdominal abscesses, the *bft* gene was detected: one of them carried the *bft*-1 isoform, and the second harboured the *bft*-2 allele. The *cfi*A gene was identified in 2 strains (3.7%) from abdominal abscesses. The fragilysin gene was detected in 17 (11.1%) isolates from other extraintestinal specimens. 14 of these 17 isolates carried the *bft*-1 isoform, while in 3 cases the *bft*-2 allele was detected. Among the examined *B. fragilis* strains, no *bft*-3 isoform was detected. The presence of the *cfi*A gene was also more frequent (7.2%) among the *B. fragilis* strains originating from other infection sites. Among 22 *B. fragilis* isolates from the faeces of healthy persons, only 1 (4.5%) harboured the fragilysin gene, and this was the *bft*-1 allele. 3 of 38 (7.9%) isolates from diarrhoeal faeces carried the *bft* gene, while only 1 carried the *cfi*A gene (2.6%). In 2 cases, *bft*-2 isoforms were detected, and 1 isolate was *bft*-1 positive.

Among the Hungarian *B. fragilis* strains, the coexistence of *bft* and *cfi*A strains could not be observed, while strain R19811 carried the *bft* and *cfi*A gene as well. In this strain, the *bft*-1 isoform was detected on PCR-RFLP. During further analysis, the strain proved resistant to many antibiotics, e.g. as penicillin G, amoxicillin/clavulanic acid, cefoxitine, clindamycin and metronidazole. In spite of the metronidazole resistance, the *nim* gene, possibly responsible for most metronidazole resistance mechanisms, could not be detected by using nim-3 and nim-5 primer pair. Because of the presence of the *cfi*A gene, the IS elements IS*614*, IS*942*, IS*1168*, IS*1169* and IS*4351* were tested; on the supposition that the occurrence of these sequences may contribute to the development of a carbapenem resistance mechanism. Of the examined IS only IS*614* proved positive by the PCR method. The sequence of the IS*614* PCR product was similar to that of IS*614*B,

but the detected sequence contained several point mutations. Analysis of the upstream region of the *cfi*A gene revealed IS*614* on use of the Up2 and G primers. The presence of IS*614* in this region may increase the expression of the *cfi*A gene as a promoter. In numerous earlier studies, the presence of the *cfi*A gene was never observed together with the *cep*A gene, because *cfi*A-positive and *cep*A-positive or *bft*-positive strains belong in two genetically distinct groups. In strain R19811, no amplification product was detected with cepA-1 and cepA-2 primer pair. This suggests that the strain originally belonged in the *cfi*A-positive group; in spite of this, it carries the *bft* gene as well.

B. fragilis strain R19811 contained a plasmid with an estimated molecular weight of 9.1 kb on the use of *Hind*III restriction enzyme. By Southern blot analysis, we tried to localize the *cfi*A gene and IS*614*B on the detected plasmid, but the hybridization experiment revealed that this plasmid did not carry the *cfi*A gene and IS*614*B. Two homology groups of *B. fragilis* can be distinguished by using ERIC PCR typing: the first group is characterized by the presence of the *cfi*A gene, these strains were *bft*-negative, while the strains in the second group carried only the *bft* gene. ERIC PCR typing suggested that R19811, which harboured the *bft* and *cfi*A gene, may be related to the first group, which contained only *cfi*A-negative strains.

5. Discussion

5.1. Studies involving *C. difficile*

5.1.1. Detection of toxins A and B, and binary toxin genes by PCR; comparison of data obtained with the cell cytotoxicity assay

During the past few years, the structure of the *C. difficile* binary toxin was described completely, but few data are available on its role in the pathogenesis of human gastrointestinal disease. However, some studies have revealed that *C. difficile* binary toxin-producing strains cause severe diarrhoea in farm animals, mainly horses, in many cases these infections proving lethal. Our studies suggest that in Hungary the prevalence of binary toxin-producing strains (2.5% of all strains, 3.5% of toxigenic strains) differs considerably from international data, e.g. a representative study indicated a prevalence of binary toxin-positive strains among toxigenic strains of 6.4% in the UK, as compared with 21.8% in Italy, 8.3% in Poland, and 17.9% in Kuwait. The data may be explained by differences in the numbers of studied isolates or the geographical distribution of the different clones. Moreover, in some publications the reason for observed high prevalence of such strains was that the strains examined originated from an outbreak caused by binary toxin-producing strains, or the studied isolates were selected from a strain collection. When the number of studied strains was low, the occurrence of binary toxin-positive isolates was high (ranging from 8.3% to 23.5%); when large *C. difficile* collections were examined, the estimated prevalence ranged from 1.6% (Japan) to 5.5% (ARU Collection, Cardiff, UK). Interestingly, two binary toxin-positive isolates observed during the present survey originated from outpatients with no previous history of hospitalization, while the third strain was isolated from diarrhoeal faeces of a hospitalized patient. Detection of the cytotoxic property of this toxin was not possible on the HeLa cell line, because of the presence of the major toxins. In these cases, therefore the presence of the binary toxin was clarified via the Western blot or the ADP-ribosyltransferase activity. However, the majority of publications dealing with binary toxin-producing strains showed that, if the binary toxin gene was present, ADP-ribosyltransferase activity or the presence of this toxin could be detected. Only one study

reported that, despite the detection of binary toxin genes by PCR, complete toxin could not be detected. In our study, the examined binary toxin-positive, toxin A and B-positive isolates suggest that this toxin variant form may be responsible for certain cases of community-acquired diarrhoea and, on the basis of three clinical pictures, we presumed that binary toxin-producing strains may cause more severe disease than the toxin A/B-positive strain itself. We therefore regard toxin detection in diarrhoeal faeces in the community and further epidemiological examinations of Hungarian strains as of high importance. The characterization of binary toxin-positive isolates and further patients from whom binary toxin-positive strains can be isolated may clarify the pathogenic role of binary toxin. During this study period, no toxin A-negative and toxin B-positive isolates could be detected. At the same time, in the last few years many study groups have described these strains from severe nosocomial infections or outbreak situations. It seems that the presence of these strains has not yet been characteristic among Hungarian *C. difficile* strains. In spite of this, because of the increasing number of toxin variant strains in various European countries, the testing of toxin A and B production together is necessary to set up correct microbiological diagnosis.

5.1.2. Results of *C. difficile* ribotyping by PCR

During the past 10 years, numerous molecular typing methods have been developed to differentiate in particular between nosocomial *C. difficile* strains. Some of these can provide useful information, especially during hospital outbreaks. In each case, the most important expectations are that the methods applied should be discriminative, reproducible, simple and rapid. PFGE is accepted as the gold standard technique for the typing of bacterial strains, but the majority of *C. difficile* isolates can not be typed because of the degradation of the DNA; moreover, this method is rather labour-intensive and time-consuming. Thus PCR ribotyping, which is convenient from every aspect, is one of the most frequently used methods for the epidemiological investigation of *C. difficile*.

In 2001, 65 *C. difficile* isolates from the University Hospital of Szeged were typed in cooperation with the ARU (Cardiff, UK) by PCR ribotyping. In that survey, 57 strains were isolated from diarrhoeal faeces and 8 from different clinical specimens, e.g. wound, bile and intra-abdominal samples. In that study period, binary toxin-producing isolates were not observed among the strains originating from Szeged University hospital. At that time, the examined strains belonged in 15 different ribotypes; the most frequent type, ribotype 087, accounted for 39% of all the examined strains, but more recently this ribotype is found less frequently (1 isolate in South Hungary and 1 in West Hungary). A number of ribotyping surveys in various countries have also demonstrated different distributions of ribotypes. For example, type 001 was detected in the majority of UK hospital infections (55%), while only 7.5% of this type caused community-acquired infections in England and Wales. In Kuwait, PCR ribotypes 097 and 078 accounted for ~40% of all isolates in the intensive-therapy units. The ribotypes of *C. difficile* strains from three parts of Hungary have been compared with the results of earlier international and national ribotyping studies. Both regional and international differences were observed as concerns the distributions of the various ribotypes, and changes in the prevalence of the different ribotypes in time were also apparent. In consequence of the increasing numbers of hospital infections and hospital outbreaks caused by *C. difficile*, the introduction of typing methods in ever more countries is essential in order to

establish a database relating to the spread of the most frequent epidemic *C. difficile* strains. Such a database would be of great help in the future demonstration of outbreaks and in the distinction of recurrences and reinfections.

5.2. Studies involving *B. fragilis*

5.2.1. Detection of ETBF strains in various clinical samples by using PCR and cell cytotoxicity assay; examination of the coexistence of the *bft* and *cfi*A genes

As *B. fragilis* is the most frequent opportunistic anaerobic pathogen in various infections, numerous research groups have tried to determine the main pathogenecity factor and its prevalence in different clinical specimens. The average occurrence of the *bft* gene among *B. fragilis* isolates from different geographical areas of Hungary was found to be 8.7%. The most frequent isoform of the *bft* gene was the *bft*-1 allele (71%), followed by *bft*-2 (29%). It seems that the *bft*-3 isoform is not characteristic among Hungarian *B. fragilis* strains, similarly as in other European countries. Previous studies have demonstrated that the prevalence of ETBF strains differs from country to country and from sample to sample. In Europe, the frequency of the *bft* gene or BFT from extraintestinal and blood isolates varies from 9% to 26% (e.g. Germany 9%, Italy 11.5%, Hungary 20.3%, Belgium 26.2%). In Hungary, an earlier survey indicated that BFT production was more frequent (40%) among *B. fragilis* isolates from diarrhoeal faeces, while in Sweden the frequency of ETBF in adult patients with diarrhoea was reported to be 26.8%. The relatively frequent occurrence of ETBF isolates originating from diarrhoeal patients in Hungary may be accounted for by the selective testing of diarrhoeal faeces. If the diarrhoeal faeces proved negative for all other frequent enteropathogenic microorganisms, such as *Salmonella* sp., *Shigella* sp., *Yersinia* sp., enteropathogenic *E. coli* and *Campylobacter* sp., the BFT production of *B. fragilis* isolates was examined on the HT-29 cell line. However, we consider, that it is difficult to determine the pathogenic role of ETBF in adult diarrhea, as the rate of ETBF carriage in healthy adults has been reported to be quite high (from 9% to 15%).

The literature data and our own results indicate that the *bft* gene is frequent among *B. fragilis* isolates from blood cultures and different extraintestinal samples. This observation and the toxic effect on the intestinal epithelium suggest that fragilysin-producing strains may escape and translocate more easily from the intestine. This hypothesis was supported by many *in vitro* experiments, which clarified that fragilysin can cleave E-cadherin, the most important structural component between intestinal cells. If the zonula occludens was damaged, alterations in the intestinal epithelial cell cytoskeleton, and loss of microvilli could be observed. At the same time, the opened space between the intestinal cells provides unrestricted access to most bacteria. However, the statistically calculated results of *Foulon et al.* suggested that BFT production is not a significant virulence factor in *B. fragilis* infections.

Our results and those of *Kato et al.* revealed that most extraintestinal isolates carried the *bft*-1 isoform, the second most frequent isoform being *bft*-2; however, in Japan 13.1% of extraintestinal isolates carried the *bft*-3 allele, whereas we have not detected this isoform in Hungary. It seems that the prevalence and geographical distribution of the *bft* isoforms differ considerably; this may be explained by the different modes of molecular evolution of the ETBF strains.

5.2.2. Analysis of *bft*- and *cfi*A positive *B. fragilis* and ERIC typing of the examined strains

In an analysis of Hungarian strains and previous, international surveys, the *bft* alleles were never found together with the *cfi*A gene. However, one strain originating from the UK has now been found to carry both the *bft* and the *cfi*A gene. This was the first case when coincidence of these two genes could be observed. This strain was isolated from a blood culture in London, the patient suffering from severe pancreatitis after gallstone removal. In spite of piperacillin/tazobactam, amikacin, metronidazole and imipenem treatment, the general condition of this patient did not improve. Antibiotic susceptibility testing suggested linezolid therapy, during which fungaemia caused by *C. guilliermondii* developed. However, a general improvement in the patient's condition was observed. The isolation of *B. fragilis* from an intra-abdominal sample and blood culture was again unsuccessful. Analysis of the *bft* gene in this strain by using PCR-RFLP revealed the *bft*-1 allele. This type is predominant among extraintestinal isolates, and also more common in strains isolated from adult faeces. Moreover, in some cases, double copies of the *bft*-1 type have been observed, these strains showing higher cytotoxic activity than strains carrying *bft*-1 alone or strains harbouring *bft*-2 or *bft*-3 isoforms. Analysis of the *cfi*A and *cep*A genes and ERIC typing pattern indicated that the strain originally carried the *cfi*A gene and acquired the *bft* gene only later.

We found that the overall prevalence of the *cfi*A gene was 5.1%, which is close to the result from this laboratory in 2000 (5.7%). The findings from other countries, e.g. France (2.4%), the UK (6.9%) and Japan (1.9% in 1987-1988, and 4.1% in 1992-1994), are reasonably similar. In *B. fragilis* strain R19811, the imipenem MIC (32 μg/ml) was greater than the current NCCLS breakpoint for resistance (≥16 μg/ml), and an IS element was detected in the upstream region of the *cfi*A gene. This additional sequence may provide a promoter region for the expression of the *cfi*A gene. On the basis of earlier results, this hypothesis was suggested by *Kato et al.*, who showed that IS*614*, IS*612*, IS*613* and IS*615*, which belong in the IS*4* family, may contribute to the development of antibiotic resistance in *B. fragilis* strains. During sequence analysis of the detected IS*614* PCR product, this insertion element showed sequence similarity with IS*614*B, but several point mutations could be observed in the obtained sequence. This strain proved resistant to metronidazole; the MIC was 64 μg/ml, but the *nim* gene could not be detected with the nim-3 and nim-5 primer pair. In spite of a reduced annealing temperature (52 °C) to show incidental sequence variation in the *nim* gene, PCR product could not be detected with these primer pair. Further investigations are necessary to clarify the possible resistance mechanism.

Gutacker et al. concluded that the *cfi*A and *bft* genes are distributed in distinct DNA homology classes of the *B. fragilis* population. Through multilocus enzyme electrophoresis, ETBF strains were allocated to division I, where occurrence of the *cep*A gene is characteristic, while *cfi*A-positive strains were grouped into division II, this cluster not containing strains carrying the *bft* or *cep*A gene. Similarly, two DNA homology classes were determined by using RFLP, DNA-DNA hybridization and 16S rRNA sequence analysis. Among Hungarian *B. fragilis* strains, which carried only the *bft* or *cfi*A gene, these two homology groups were separated by using ERIC PCR typing. Strain R19811, harbouring both *bft* and *cfi*A genes, gave a similar ERIC PCR pattern to that of the only-*cfi*A-positive strain, suggesting that this strain first carried the *cfi*A gene, and then gained the *bft* gene. This hypothesis was supported by the result of the *cep*A

PCR: the endogenous class A β-lactamase gene could not be detected in this strain. This is also characteristic in the second homology group, which contains only *cep*A-negative and *bft*-negative strains. The *cfi*A and *bft* genes and other resistance mechanisms may contribute to enhance the pathogenecity potential of this strain, and these factors together lead to severe and lethal infection. Data on the prevalence of the major *C. difficile* toxin genes, *bft* and *cfi*A among Hungarian *B. fragilis* isolates may help to clarify their pathogenic potential. The results of *C. difficile* ribotyping and ERIC typing of *B. fragilis* strains may help explain the origin and spread of these strains, and draw attention to the importance of epidemiological investigations of anaerobic bacteria.

6. CONCLUSIONS

During my studies, 118 *C. difficile* strains isolated from faecal specimens were examined:

1. 85 isolates carried both the *tcd*A and *tcd*B genes; in 33 cases, both toxin A and B genes could not be detected.
2. 3 strains harboured binary toxin genes; 2 strains were isolated from severe community-acquired diarrhoea, while 1 strain originated from nosocomial diarrhoea. These were the first binary toxin-producing strains described in Hungary. These strains can produce toxin A and B, and thus a cytotoxicity assay for detection of the binary toxin was not available.
3. On analysis of the 3' end of the toxin A gene, deletion or insertion could be detected with the applied primer pair.
4. Binary toxin-positive strains produced the usual CPE on the HeLa cell line. The same morphological alteration was observed for the toxin A/B-positive strains.
5. PCR ribotyping revealed different distributions of *C. difficile* ribotypes in three Hungarian regions.
6. Changes in the distribution of *C. difficile* ribotypes in time were demonstrated.
7. The ribotyping results demonstrated a higher prevalence of ribotypes 012 among inpatients than among outpatients. In most cases, significant statistical differences in frequency were observed between various ribotypes and patient groups.

On analysis of 275 *B. fragilis* strains isolated from various clinical specimens:

8. The overall frequency of the *bft* gene was 8.7%; the presence of *bft*-positive isolates was characteristic in blood specimens or other infection sites (diabetic ulcers, pelvic abscesses, etc.).
9. Most fragilysin-producing strains carried the *bft*-1 isoform, while the *bft*-2 isoform could be detected in 29.2%. The presence of the *bft*-3 allele was not characteristic.
10. The coincidence of *bft* and *cfi*A was not detected among the examined Hungarian *B. fragilis* strains. The overall prevalence of *cfi*A-positive isolates in various clinical specimens was 5.1%.
11. Coincidence of the *bft* and *cfi*A genes was observed in a multiresistant *B. fragilis* strain originating from the UK. This was the first isolate in which these two genes were present together. ERIC typing and *cep*A PCR suggested that this strain originally carried the *cfi*A gene and later gained

the *bft* gene as well. In the upstream region of the *cfi*A gene, the IS*614* element was detected, which may contribute to enhance the expression of the *cfi*A gene. In spite of the observed metronidazole resistance in this case, the *nim* gene could not be detected with the applied primer pair. Southern blot analysis revealed that the 9.1 kb plasmid detected in this strain did not carry the *cfi*A gene and the IS*614* element.

12. The ERIC typing results revealed that the *cfi*A- and *bft*-positive strains belong in different DNA homology classes.

LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THIS THESIS

Full papers cited in the thesis

I. **Terhes, G.,** E. Urbán, J. Sóki, K. A. Hamid, and E. Nagy. Community-acquired *Clostridium difficile* diarrhea caused by binary toxin, toxin A, and toxin B gene-positive isolates in Hungary. *Journal of Clinical Microbiology* (2004) **42:**4316-4318. IF: 3.439

II. **Terhes, G.,** J. S. Brazier, E. Urbán, J. Sóki, and E. Nagy. Distribution of *C. difficile* PCR ribotypes in regions of Hungary. *Journal of Medical Microbiology* (2006) 55:279-282. IF: 2.484

III. **Terhes, G.,** E. Urbán, E. Nagy. *Clostridium difficile* törzsek isolálása, toxintermelésének vizsgálata és ribotípusának meghatározása. *Infektológia és Klinikai Mikrobiológia* (2005) **12:**37-43. IF:-

IV. **G. Terhes,** J. Sóki, E. Urbán, E. Nagy. *bft*-1 is the most prevalent isoform among enterotoxigenic *Bacteroides fragilis* strains in Hungary. (submitted for publication).

Full papers related to the subject of the thesis

I. Urbán, E., A. Tusnádi, **G. Terhes,** and E. Nagy. Prevalence of gastrointestinal disease caused by *Clostridium difficile* in a university hospital in Hungary. *Journal of Hospital Infection* (2002) **51:**175-178. IF: 1.909

II. Urbán, E., **G. Terhes,** E. Nagy. Milyen gyakran kell számolni fekvőbeteg intézetben *Clostridium difficile* által okozott hasmenéssel? *Infektológia és Klinikai Mikrobiológia* (2000) **7:**136-139. IF:-

Abstracts related to the subject of the thesis

I. **Terhes, G.,** J. Sóki, E. Urbán, and E. Nagy. Prevalence of *bft* isoforms in *Bacteroides fragilis* strains of different origins in Hungary. Abstracts: Anaerobe 2004. The 7th Biennial Congress of the Anaerobe Society of the Americas, Annapolis, Maryland, USA, 19-21 July 2004. *Anaerobe* (2005) **11:**14.

II. Soki, J., **G. Terhes**, V. O. Rotimi, E. Urbán, and E. Nagy. Estimation of potential risk of emerging carbapenem and metronidazole-resitant *Bacteroides*. Abstracts: Anaerobe 2004. The 7th Biennial Congress of the Anaerobe Society of the Americas, Annapolis, Maryland, USA, 19-21 July 2004. *Anaerobe* (2005) **11:**52.

III. **Terhes, G.,** E. Urban, J. S. Brazier, J. Soki, and E. Nagy. Epidemiological examination of *Clostridium difficile* from different parts of Hungary. Abstracts:

15th European Congress of Clinical Microbiology and Infectious Diseases. 2-5 April 2005. Copenhagen, Denmark. *Clinical Microbiology and Infection* (2005) **11** (Suppl. 2)**:**487.

IV. **Terhes, G.,** J. Soki, K. Ago, E. Urban, and E. Nagy. Detection of BFT, the isoforms of the enterotoxin gene and *cfi*A gene in *Bacteroides fragilis* isolates of different origins. Abstracts: 14th European Congress of Clinical Microbiology and Infectious Diseases. 1-4 May 2004. Prague, Czech Republic. *Clinical Microbiology and Infection* (2004) **10** (Suppl. 3)**:**253.

V. **Terhes, G.,** E. Urbán, J. Sóki, and E. Nagy. Detection of binary toxin genes (*cdt*A, *cdt*B) of *Clostridium difficile* isolates from different parts of Hungary by PCR method. FICDS First International *Clostridium difficile* Symposium, 5-7 May 2004. Kranjska Gora, Slovenia.

VI. **Terhes, G.,** E. Urbán, J. Sóki, and E. Nagy. Detection of toxin genes (*tcd*A, *tcd*B, binary toxin) of *Clostridium difficile* isolates from different parts of Hungary by PCR method. 14th International Congress of the Hungarian Society for Microbiology, 9-11 October 2003. Balatonfüred, Hungary.

VII. **Terhes, G.,** J. Sóki, K. Ágó, E. Urbán, and E. Nagy. Detection of *bft* and *cfi*A genes of *Bacteroides fragilis* isolates of different origins by PCR methods. 3rd World Congress on Anaerobic Bacteria and Infections, 7-9 May 2003.Glasgow, Scotland, UK.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Prof. Dr. Elisabeth Nagy, for her continuous support and guidance throughout this study.

I express my deepest thanks to Dr. Edit Urbán and Dr. József Sóki for their help and support, and for imparting their valuable knowledge to me.

I am especially grateful to Dr. Judith Deák and the staff of the virology unit for helpful cooperation, and the excellent preparation of different cell lines.

I thank Dr. Jon S. Brazier and Prof. Ian Poxton for permission to carry out the ribotyping method, and S-layer typing in their laboratories.

I am grateful to Drs. Adlan Elmardi, Galina Riszkulova, Mária Tömöry, Anna Tusnádi, Mária Vollain, Kanjo Abdul Hamid, Zoltán Lajos, Cecília Miszti and Lenke Szikra for kindly providing *C. difficile* and *B. fragilis* strains.

I am grateful to Dr. Edit Hajdú, Dr. Eleonóra Fodor, Csilla Rátkai, Dr. Ferenc Somogyvári, Ilona Dóczi, Mariann Ábrók, Edit Tenkes and Betarix Kele for their helpful cooperation, and their support.

I am grateful to Anita Bönde and Ensieh Akhavansharif for their excellent technical assistance and cooperation.

I wish to thank all my colleagues and staff members at the Institute of Clinical Microbiology for creating a supportive, pleasant environment, and technical assistance contributing to this work.

I would like to thank my mother, my sister, my niece and my nephew for their love and support.

This work was supported by an Hungarian Eotvos Scholarship (Gabriella Terhes, 2003/2004), grants TO32385 and TO37475 from the Hungarian National Research Foundation (OTKA).