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ANTIMICROBIAL RESISTANCE

# Prevalence of the carbapenemase gene (*cfiA*) among clinical and normal flora isolates of *Bacteroides* species in Hungary

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**The carbapenemase gene (*cfiA*) was detected in 4 (5.7%) of 70 clinical isolates of *Bacteroides fragilis* from different parts of Hungary. Among 24 other *Bacteroides* species isolated from infectious processes or from normal faecal flora, none was *cfiA*-positive. The MIC of imipenem and meropenem for all *cfiA*-positive *B. fragilis* isolates was ≤0.25 mg/L, but 17% of the *B. fragilis* and 46% of the non-fragilis *Bacteroides* isolates exhibited reduced susceptibility to imipenem (MICs 0.5–2 mg/L). Only one of these isolates produced increased levels of β-lactamase. No difference was observed in the outer-membrane proteins of *B. fragilis* isolates that harboured the *cfiA* gene and those with reduced susceptibility to imipenem.**

## Introduction

Members of the genus *Bacteroides* (*B. fragilis* group) are frequently isolated from infections after abdominal or gynaecological surgery [1]. These infections are usually polymicrobial and demand the use of potent antibiotics, covering both aerobic and anaerobic pathogens. Increasing resistance of anaerobic bacteria to the antimicrobial agents used for the treatment of anaerobic infections, and differences in the rates of resistance between various hospitals and countries have been reported [2, 3]. Resistance to β-lactam antibiotics in anaerobic bacteria involves the production of β-lactamases, alterations in the penicillin-binding proteins, or the reduced penetration of the antibiotics through the bacterial outer membrane [4]. In *Bacteroides* spp., resistance to β-lactam agents is most often mediated by chromosomal class A β-lactamases with predominant cephalosporinase activity, which are virtually ubiquitous in *B. fragilis* group isolates [5], although the amount of the enzyme varies greatly from isolate to isolate. These enzymes are inhibited by clavulanate, tazobactam and sulbactam and do not hydrolyse cephamycins or carbapenems. Several studies [6–9] have suggested that 0.7–3% of *B. fragilis* strains produce class B carbapenem-hydrolysing metallo-β-lactamases, which are inhibited by ethylenediaminetetraacetic acid (EDTA), but not by the clinically used β-lactamase inhibitors. The *cfiA* gene coding this enzyme has been cloned and sequenced. It may be 'silent' or expressed to various degrees, resulting in a wide range of levels of carbapenem resistance. Some strains harbouring the *cfiA* gene appear sensitive to carbapenems on conventional testing, but can convert to high-level resistance following antibiotic pressure [8]. Clinically relevant *B. fragilis* strains, with or without the presence of the *cfiA* gene, have been reported from different countries to be resistant to imipenem (MIC ≥16 mg/L), or to display only a decreased susceptibility (MIC ≥1 mg/L) [9, 10]. The present study looked for the presence of the *cfiA* gene by the PCR in clinical isolates of *B. fragilis* collected at three different centres in Hungary. A limited number of strains belonging to other species of *Bacteroides*, obtained from clinical samples or from the normal faecal flora, were also tested.

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## Materials and methods

### *Bacterial strains and plasmid*

Seventy randomly selected, clinically significant *B. fragilis* isolates and 24 isolates of other *Bacteroides* species (including 10 isolates obtained from faeces of healthy subjects) were collected during 1998 from a large university hospital in south-east Hungary (39 isolates), a large community hospital in the central part of the country (21 isolates) and a small hospital in western Hungary (10 isolates). Isolates were identified by conventional tests or by the Rapid ID 32A system (bioMérieux). *B. fragilis* NCTC 9344 (carbapenem-

sensitive) and *B. fragilis* TAL3636 (a metallo-β-lactamase producer), kindly provided by R. Edwards (Nottingham University), were included as controls. The pJST241 plasmid, carrying the cloned *cfiA* gene [8], was obtained from M. H. Malamy (Boston, MA, USA). The Qiagen Mini Plasmid Purification kit (Qiagen, Hilden, Germany) was used for plasmid isolation.

### *Antibiotic resistance determination*

MICs 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 manufacturer's instructions. MICs were read after incubation for 48 h in an anaerobic environment (Bactron, Shell Lab., Cornelius, USA).

### *Determination of β-lactamase activity*

β-Lactamase activity was determined quantitatively with (0.10 mM) nitrocefin in 50 mM sodium phosphate buffer (pH 7.0, 37°C) by a spectrophotometric method [11]. One unit of β-lactamase was defined as the amount that formed 1.0 μmole of product/min under the given conditions.

### *Investigation of outer-membrane proteins*

Outer-membrane proteins (OMPs) of selected *B. fragilis* isolates (four strains that were *cfi*A-positive, but imipenem susceptible and four that were *cfi*A-negative, but for which the MIC of imipenem was 1–2 mg/L) were examined by a method based on that described by Carlone *et al.* [12]. The OMPs were solubilised by boiling with 50 μl of Laemmli sample buffer for 5 min and separated by SDS-PAGE.

### *Detection of the cfiA gene by PCR*

Bacterial cells from the surface of BHI agar plates were suspended in water and boiled for 10 min. The supernates of the centrifuged suspensions (2 min at 10 000 rpm) were used as template DNA. Reaction mixtures each contained 5 μl of 10-fold concentrated reaction buffer (Sigma or USB), 1 μl (2.5 mM) each of dATP, dCTP, dGTP and dTTP (Sigma), 1 μl (35 pmoles) of each primer, 5 μl of template DNA, 33 μl of sterile water and 1 U of Taq polymerase (Sigma). The primers had the nucleotide 557–582 sequence 5′-TCC ATG CTT TTC CCT GTC GCA GTT AT-3′ and the complementary 1266–1285 sequence 5′-GGG CTA TGG CTT TGA AGT GC-3′ [8]. The reaction mixtures were incubated for 40 cycles in a programmable heating block (GeneAmp, PCR System 9600, Perkin Elmer, Norwalk, CT, USA) for 1 min at 92°C, 2 min at 50°C and 2 min at 72°C with a final extension of 10 min. PCR products were visualised on agarose gels containing ethidium bromide under UV light and their sizes were compared with those of a mol. wt 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 agarose 1% gels and transferred to nylon membranes (Amersham, UK) by capillary transfer. Radioactive labelling of the probe was performed with the Megaprime random priming kit (Amersham) under the conditions recommended by the supplier. Pre-hybridisation at 65°C for 1 h was followed by hybridisation at 65°C overnight in 5× concentrated saline sodium citrate (SSC) buffer and 5× concentrated Denhardt's solution containing sodium dodecyl sulphate (SDS) 0.5% w/v and salmon sperm DNA 100 μg/ml. Radioactively labelled probe DNA with an activity of *c.* $10^7$ cpm was added to the hybridisation solution after pre-hybridisation. The filters were washed twice in double-strength SSC and SDS 0.1% w/v at 65°C for 15 min, and twice in 0.2× SSC and SDS 0.1% w/v at 65°C for 15 min. Damp filters were packed in Saran Wrap and were exposed to X-ray films at −70°C with an intensifying screen.

### *Nucleotide sequencing*

Before nucleotide sequencing, the amplified PCR sample was precipitated with three volumes of 7.5 M ammonium acetate and absolute ethanol (1:5 v:v) at room temperature to remove unincorporated primers. DNA sequencing was performed with an automated sequencer (ABPrism model 373, Applied Biosystems) with an AmpliTaq FS DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The DNA sequence obtained was compared with those of known carbapenemase genes from *Bacteroides* spp. with the BLAST client programme accessible from the Internet at the National Center for Biotechnology Information.

## Results and discussion

The MICs of imipenem for 70 *B. fragilis* and 24 non-fragilis isolates ranged from ≤0.06 to 2 mg/L. No resistant strain was observed according to the NCCLS breakpoint criterion (MIC ≥16 mg/L) [13]. However, 12 *B. fragilis* isolates (17%) and 11 non-fragilis isolates (46%) exhibited reduced susceptibility to imipenem (MICs 0.5–2 mg/L). The *cfiA* gene was detected in four *B. fragilis* isolates and in none of the non-fragilis isolates by PCR. Southern blotting of the PCR products revealed homology to the pJST241 probe in *B. fragilis* isolates 20, 72 and 98. The PCR product of *B. fragilis* isolate 22 did not hybridise, but sequence analysis of the product showed that it bore *c.* 96% homology to known *cfiA* genes, indicating that it represents a carbapenemase gene fragment.

**Table 1.** MICs of carbapenems and the presence of the *cfiA* gene in selected *B. fragilis* isolates obtained from clinical specimens

| Isolate | MIC (mg/L) of | | *cfiA* | β-lactamase activity (U/min) |
|---|---|---|---|---|
| | imipenem | meropenem | | |
| *B. fragilis* 22 | 0.06 | 0.125 | + | 0.0175 |
| *B. fragilis* 20 | 0.06 | 0.06 | + | 0.0028 |
| *B. fragilis* 98 | 0.125 | 0.25 | + | 0.0004 |
| *B. fragilis* 72 | 0.25 | 0.25 | + | 0.0109 |
| *B. fragilis* 91 | 1.0 | 0.5 | − | 0.0140 |
| *B. fragilis* 76 | 1.0 | 0.125 | − | 0.0087 |
| *B. fragilis* 68 | 2.0 | 1.0 | − | 2.9540 |
| *B. fragilis* 54 | 2.0 | 2.0 | − | ND |
| *B. vulgatus* 38 | 1.0 | 1.0 | − | 0.0152 |
| *B. thetaiotaomicron* 55 | 2.0 | 2.0 | − | 0.0185 |
| *B. ovatus* 42 | 2.0 | 2.0 | − | ND |

ND, not detected.

The MICs of imipenem and meropenem for the four *cfiA*-positive isolates were 0.06–0.25 mg/L (Table 1). Very low β-lactamase activities were measured in these isolates. β-Lactamase activities were also low for three *B. fragilis* and three *Bacteroides* non-fragilis isolates, for which the MIC of imipenem was 1 or 2 mg/L. One strain (*B. fragilis* 68), for which the MICs of imipenem and meropenem were 1 and 2 mg/L, respectively, was found to have high β-lactamase activity. PAGE analysis of the OMPs of the *B. fragilis* isolates with elevated MICs and those that were fully susceptible, but harboured the *cfiA* gene, revealed no difference (results not shown). Similar results have been described by Edwards and Greenwood [14].

No imipenem-resistant isolate has yet been reported in Hungary (the MIC90 was 0.5 mg/L in 1992 and 1 mg/L in 1992–1993), despite the fact that imipenem or meropenem are increasingly used for treatment of severe infections involving aerobic and anaerobic bacteria [10, 15]. Low rates of resistance to imipenem among *B. fragilis* strains have been found in France [2] and in the USA [16], whereas in Japan, where imipenem is widely used, 2.4, 4.5 and 10.5%, respectively, of the *B. fragilis*, *B. thetaiotaomicron* and *B. distasonis* isolates tested were resistant to imipenem [17]. However, the resistance breakpoint used in the Japanese study (6.25 mg/L) was lower than that recommended by NCCLS guidelines (16 mg/L). High-level resistance to imipenem was also found to be rare among *B. fragilis* isolates collected in Nottingham, UK [9], although 12% of 175 *Bacteroides* isolates from clinical specimens exhibited a reduced susceptibility to imipenem (MICs 0.5–2 mg/L, i.e., concentrations up to 50 times higher than those for 'normal' sensitive strains [9]). In the present study, MICs of imipenem for 17% of the *B. fragilis* and 46% of the non-fragilis isolates were ≥10-fold higher than those for fully sensitive strains. The percentage of clinical isolates of *B. fragilis* (5.7%) found to possess the *cfiA* gene in the present study is substantially more than the rate reported from France (2.4%) [6], but similar to that observed by Edwards *et al.* [9].

All four *cfiA*-positive strains were obtained from patients in a large university hospital. Although these isolates would be classified as sensitive by conventional breakpoint criteria, they have the potential to convert to a high level of metallo-β-lactamase expression by acquisition of an insertion element carrying an efficient promoter of the *cfiA* gene [18].

Few clinically relevant *Bacteroides* strains have been reported world-wide to harbour the *cfiA* gene or to express metallo-β-lactamase production, resulting in carbapenem resistance and possible failure of therapy. However, Fang *et al.* [19] found the *cfiA* gene in faecal isolates of *B. fragilis* and species other than *B. fragilis* with high-level production of metallo-β-lactamase have also been isolated [20]. Careful monitoring of changes in the imipenem resistance of *Bacteroides* strains isolated from clinical samples and from normal faecal flora, together with screening for the *cfiA* gene, are important to follow the emergence and spread of carbapenem resistance in different parts of the world.

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# II.

# Az első hazai carbapenemase termelésen alapuló imipenem rezisztens *Bacteroides fragilis* törzsek genetikai vizsgálata

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## Összefoglalás

*Szerzők beszámolnak az első hazai bizonyítottan carbapenemase enzimet termelő, imipenem rezisztens* Bacteroides fragilis *törzsek vizsgálatáról. Az egyik törzs egy 22 éves fiatalember hasüri váladékából került izolálásra a gangraenás appendicitis miatt történt hasi műtét során. A másik törzset egy kutya prostata tályogából izolálták. Mindkét törzs magasfokú rezisztenciát mutatott imipenemmel szemben (MIC: >256 mg/l). Megfelelő specifikus primerekkel a szerzők PCR módszert használva igazolták a rezisztenciáért felelős* cfiA *gén jelenlétét, valamint expressióhoz szükséges IS942 promoter meglétét. A magasfokban carbapenem rezisztens* B. fragilis *törzsek hazai megjelenése a humán és az állati eredetű klinikai anyagban, az előzőekben igazolt „silent"* cfiA *gént hordozó törzsek mellett azt jelzi, hogy a* B. fragilis *törzsek imipenem érzékenysége nem tekinthető száz százalékosnak, így a súlyos esetekből izolált törzseknél az antibiotikum érzékenység vizsgálata ajánlott.*

A carbapenemek a beta-lactam antibiotikumok leghatékonyabb csoportját képviselik, melyek igen nagy affinitást mutatnak a penicillin kötő fehérjékhez és rendkívül rezisztensek a legtöbb beta-lactamase-val szemben. A Gram-negatív és Gram-pozitív aerob és anaerob baktériumok legnagyobb részével szemben hatékonyak, így empiricusan jól alkalmazhatók anaerob vegyes infectiók esetén. Mindössze néhány, elsősorban nosocomialis infectióban szerepet játszó species (*Stenotrophomonas maltophilia, Burkholderia cepacea, Flavobacterium*), illetve néhány *Bacteroides fragilis* törzs esetében igazolták a különleges metallo-beta-lactamase termelésen alapuló carbapenem rezisztencia meglétét.

Az elmúlt években egyre több közlemény jelent meg Európa különböző országaiban súlyos infectiókból származó olyan *B. fragilis* törzsek isolálásáról, melyek hordozzák a carbapenemase termelésért felelős *cfiA* gént.[1-4] Az eddig isolált legtöbb *cfiA* gén hordozó *B. fragilis* törzs esetében a gén nem expressálódik és így az isolált törzs az in vitro rezisztencia vizsgálat során gyakorlatilag érzékenynek bizonyul imipenemmel szemben (MIC: <8 mg/l). A mért MIC érték azonban 10-20× magasabb lehet, mint a gént nem hordozó *B. fragilis* populatiónál talált érték. Az isolált *cfiA* pozitív *B. fragilis* törzsek egy részénél azonban megfigyelhető a gén aktiválódása és ilyenkor egy IS elem („insertion sequence element") mutatható ki a *cfiA* génhez viszonyítva „upstream" ~50 bp távolságra, mely tartalmaz egy olyan promoter régiót, mely a gén expressióját váltja ki és így a törzs nagymennyiségű carbapenemase enzimet termel és magasfokban rezisztens imipenemmel szemben (MIC >256 mg/l).[5] Az enzimet nagymennyiségben termelő *B. fragilis* törzsek között a két leggyakrabban megtalálható IS elem, az IS1186 és az IS942.[6]

Az anaerob törzsek reziszencia viszonyainak felmérésére laboratóriumunk évek óta rendszeresen gyűjti a különböző hazai anaerob tenyésztéssel foglalkozó laboratóriumok által, releváns klinikai anyagokból isolált anaerob törzseket. A több év óta végzett vizsgálatainkról szakmai fórumokon és közleményekben számoltunk be.[7,8] Ezen vizsgálatok során a hazai *Bacteroides* genusba tartozó törzsek imipenem MIC értéke <0,25-4 mg/l között volt mind 1992-ben, mind az 1993-94-ben gyűjtött törzsek esetében.[8] Tehát az NCCLS „guideline" alapján egyetlen törzs sem bizonyult rezisztensnek. Az 1997-98 között gyűjtött törzsek vizsgálata során 70 klinikai mintából származó *B. fragilis* törzs között PCR módszerrel 4 törzs esetében (5,7%) kimutattuk a *cfiA* gén jelenlétét, azonban a törzsek imipenem és meropenem MIC értéke 0,06 és 0,25 mg/l között volt, amihez igen alacsony beta-lactamase aktivitás társult (0,0004-0.0175 U/min) a nitrocefin módszert alkalmazva.[4]

Jelen vizsgálatunk során összesen 83, klinikai mintából (43 törzs) és normál székletmintából (40 törzs) származó *B. fragilis* törzs imipenem rezisztenciáját vizsgáltuk korongdiffúziós screening módszerrel, majd a pontos MIC értéket „E" teszttel, valamint agarhígításos módszerrel határoztuk meg. Két törzset találtunk, melyek szűkült gátlási zónával rendelkeztek és „E" test-tel >32 mg/l MIC értékűek voltak imipenemmel szemben. Az egyik isolatum egy 22 éves fiatalember hasüri váladéká-

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ból került isolálásra a gangraenás appendicitis miatt történt hasi műtét során, a másik törzset egy kutya prostata tályogából isoláltuk. Az agarhígításos módszerrel végzett MIC érték meghatározás bizonyította, hogy mind két törzs igen nagy mértékben rezisztens a carbapenemekkel szemben (MIC: >256 mg/l volt mind imipenemmel, mind meropenemmel szemben). Az érzékenységi vizsgálatokat anaerob véres agar táptalajon végeztük, az incubálás anaerob kamrában (Bactron, Shell Lab, Anglia) történt 48 órán keresztül. A magasfokú carbapenem rezisztenciáért felelős genetikai elem (*cfiA* gén) kimutatására PCR módszert alkalmaztunk előző vizsgálatainknak megfelelően.[4] Emellett elvégeztük a specifikus beta-lactamase enzim kimutatását is. 299λ hullámhosszon mértük a két törzs 24 órás BHI leves tenyészetének ultrahangozott, szűrt felülúszójának aktivitását imipenemre, mint substratra az idő függvényében.[9] Mindkét törzs a kontroll imipenem rezisztens *B. fragilis* isolatummal (TAL3636) azonos mennyiségben termelte a metallo-beta-lactamase-t, melynek aktivitása EDTA-val gátolható volt. Kíváncsiak voltunk, hogy előző vizsgálataink során *cfiA* pozitívnak talált imipenem érzékeny *B. fragilis* isolatumok,[4] illetve a most vizsgált két carbapenemase termelő isolatum rendelkezik-e és ha igen milyen IS elemmel. A *B. fragilis* törzseknél a hatékony expressióhoz elengedhetetlen ismert IS elemek, az IS1186 és az IS942 génsequentiájára specifikus primaereket használva[1] igazoltuk, hogy a *cfiA* gént nem expressáló isolatumok nem tartalmaztak IS elemet, míg a két rezisztens törzsnél az IS942 elem kimutatható volt PCR segítségével. Az IS elemek PCR detektálása során a *B. fragilis* TAL3636 (IS942) és a *B. fragilis* Bf8 (IS1186) törzset használtuk kontrollként. Fontos megjegyezni, hogy sem az emberi sem a kutya isolatum esetében előzetes antibiotikum terápia nem történt, így nem lehetett az imipenem vagy meropenem adásának selectiv hatását igazolni.

Vizsgálataink arra hívják fel a figyelmet, hogy bár nem nagy gyakorisággal, de hazánkban is megjelentek az emberi és állati infectiós folyamatból származó *B. fragilis* törzsek között a világ más országaiban már észlelt imipenem rezisztens isolatumok. A metallo-beta-lactamase termelésért felelős gén és az expressiót kiváltó IS elem magasfokú rezisztenciát biztosító enzimtermeléshez vezet. Sajnálatos módon a klinikai mikrobiológiai laboratóriumok Magyarországon nem vagy csak igen kis számban végeznek MIC érték meghatározáson alapuló rezisztencia vizsgálatot a különböző infectiókból származó anaerob isolatumok esetében. Így meglehetősen nehezen ítélhető meg, hogy országosan milyen %-ban kell jelenleg számolni a carbapenem rezisztenciával a klinikailag releváns *B. fragilis* törzsek között. Még kevésbé tudjuk, hogy a normál székletflórában milyen gyakorisággal vannak jelen a normál flóra tagjaiként a carbapenemase termelő *B. fragilis* törzsek. A carbapenem rezisztencia mellett a nemzetközi irodalom egyre többet foglalkozik a metronidazol, clindamycin, cefoxitin és a beta-lactam/beta-lactamase gátló kombinációkkal szembeni rezisztenciával a klinikai anaerob isolatumokban, ami felveti az eddigi rezisztencia vizsgálatokkal kapcsolatos hazai gyakorlat felülvizsgálatának szükségességét.

## Irodalom

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Nagy, E., Sóki, J., Edwards, R., Urbán, E., Lajos, Z., Szabó, B.: **Genetic Examination of the First Isolate of Carbapenemase Producing *Bacteroides fragilis* Strain in Hungary**

## Summary

The first report is presented here about the isolation of carbapenemase producing *Bacteroides fragilis* strains in Hungary. The first isolate was obtained from the intraabdominal cavity during operation of a 22 years old male patient suffering from gangrenous appendicitis. The second strain was isolated from the prostate abscess of a a dog. The imipenem MIC was >256 mg/l in the case of both strains. Using specific primers, the *cfiA* gene, responsible for the imipenem resistance in *B. fragilis* strains, was detected and the IS942 insertion element was also present in both strains. The appearance of the imipenem highly resistant *B. fragilis* strains in clinical materials obtained from men and dog draw the attention that imipenem sensitivity among these isolates should not be taken for garanted and highlightes the need of testing the antibiotic sensitivity of anaerobic bacteria in the everyday routine.

# III.

# Milyen gyakran kell számolni fekvőbeteg intézetben *Clostridium difficile* által okozott hasmenéssel?

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A *Clostridium difficile* obligat anaerob Gram-pozitív spóraképző pálca, mely legalább két exotoxint termel: egy A toxint, mely enterotoxin és egy B toxint, mely elsősorban cytotoxicus hatással bír.[1,2] Napjainkra a kutatások igazolták, hogy mint kórokozó, elsősorban a gastrointestinalis infekciókban játszik jelentős szerepet annak ellenére, hogy számos extraintestinalis kórképből is isolálták. Így a pseudomembranás colitisek több mint 90%-ában sikerült toxin termelő *Clostridium difficile* törzset kimutatni.[5,15] Ugyancsak igazolták kóroki szerepét az antibiotikum adását követően fellépő "antibiotic-associated diarrhea"-k jelentős részénél, ahol a gastrointestinalis tünetekért szintén a törzsek által termelt toxinok tehetők felelőssé.[8,9] A *Clostridium difficile* által okozott gastrointestinalis infekciók súlyossági foka széles skálán mozog, az asymptomaticus colonisatiótól az enyhébb-súlyosabb hasmenéseken keresztül a letalis toxicus megacolonig, esetleg colon perforatióig terjedhet. Leggyakoribb az enyhébb lefolyású, kifejezett morphologiai elváltozásokkal nem járó hasmenéses megbetegedés, azonban a nemzetközi irodalmi adatok azt mutatják, hogy növekszik a súlyosabb, a distalis colonra lokalizálódó, kifejezett morphologiai elváltozásokkal járó, gyakran letalis kimenetelű megbetegedések száma.[4,9,10,11,15] A toxin termelő *Clostridium difficile* törzsek a nosocomialis hasmenések leggyakrabban isolált kórokozói, kórházi hasmenéses járványok előidézői lehetnek, ahol a törzsek kórházi terjedése az egyes betegekről, a kórházi környezetből, a tárgyakról, az ápolószemélyzet kezéről epidemiológiai tipizáló módszerekkel jól nyomonkövethető.[4,5,7,10,12] A *Clostridium difficile* által okozott nosocomialis hasmenések praedisponáló tényezői: az idős kor, a hosszan tartó (>3 nap) hospitalisatio, az előzetes antibiotikum kezelés és az esetlegesen fennálló alapbetegségek. A *Clostridium difficile* által okozott nosocomialis hasmenések jelentős problémát jelenthetnek mind a klinikusnak, mind a higiénikusnak/epidemiológusnak, elsősorban a bizonyítottan járványos terjedés miatt, de nem elhanyagolható jelentőségű a terápia költségvonzata sem.[3,15]

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Közleményünkben saját tapasztalatainkról számolunk be tartós hospitalisatio után előforduló hasmenéses megbetegedések során kimutatott *Clostridium difficile* toxin vizsgálatainkról, összevetve azon célzott vizsgálatokkal kapott eredményeket, amikor a klinikus kérte a *Clostridium difficile* toxin vizsgálatot, a laboratóriumunk által felállított kritériumok szerint szelektált székletmintákból végzett *Clostridium difficile* toxin kimutatás eredményeivel, ahol a klinikus nem gondolt a *Clostridium difficile* kóroki szerepére.

## Anyagok és módszerek

A vizsgálatokba bevont székletminták egy részét az 1999. április 15.- 1999. december 15. közötti időszakban laboratóriumunkba, a SZTE ÁOK klinikáiról szokványos enteralis kórokozók tenyésztése céljából beküldött 1860 székletminta közül választottuk ki a székletben megtalálható szabad *Clostridium difficile* toxin kimutatás céljából. A válogatás szempontjai az alábbiak voltak: a híg, hasmenéses (esetleg véres-nyákos) széklet, melyből szokványos enteralis pathogen baktérium nem tenyészett, rota vagy adenovirus nem volt kimutatható, valamint olyan tartósan hospitalisált (>3 nap) betegtől származott a széklet, akinek az esetében a klinikus nem kért *Clostridium difficile* toxin vizsgálatot. A fenti kritériumoknak ebben az időszakban összesen 318 székletminta felelt meg, ezekből a mintákból a székletben található szabad toxin kimutatását HeLa sejttenyészeten végeztük. Értékeltük a *Clostridium difficile* toxin pozitív betegek esetében a hasmenés kialakulása és az antibiotikum adása közötti összefüggést is azon toxin pozitív betegek esetében, akiknél a klinikus feltüntette az antibiotikum adást a vizsgálatkérő lapon.

A vizsgálati anyagok másik részét laboratóriumunkba ugyanebben az időszakban a SZTE ÁOK klinikáiról származó olyan székletminták képezték, amelyek a klinikus kérésére célzottan *Clostridium difficile* toxin kimutatására érkeztek. A célzott vizsgálatra beérkezett 150 székletmintát a VIDAS (bioMérieux) *Clostridium difficile* toxin A ELFA (Enzime-Linked-Fluorescent-Assay) módszerrel dolgoztuk fel.

**Clostridium difficile *toxin kimutatása szövetkultúrán***

A székletminták gyűjtése, tárolása a standard laboratóriumi módszereknek megfelelő módon történt, a vizsgálat elvégzésére a friss székletminta a legalkalmasabb. Abban az esetben, ha nincs lehetőség a székletminta azonnali feldolgozására, illetve a vizsgálat azonnali elvégzésére, a mintákat 3 napig hűtőszekrényben (2-8 °C), vagy 1 hónapig –20 °C-on tárolhatjuk.

A mintafeldolgozás során kb. 5 g, vagy híg széklet esetén kb. 0,5 ml székletet 1:1 arányban physiologiás sóoldattal (0,85% NaCl pH 7,4) elszuszpendáltunk. A szuszpenziót Vortexeléssel homogenizáltuk, majd ezután Eppendorf centrifugában 12.000-es fordulatszámon, 5 percig szobahőn centrifugáltuk. A centrifugált széklet-felülúszót steril, fecskendőhöz csatlakoztatható membranszűrőn (MILLEX®-GV, alacsony protein kötésű, 0,22 µm pórusnagyságú) baktériummentesre szűrtük. Ezt a szűrletet –20 °C-on tárolhatjuk a szövettoxicitási próba beállításáig, legfeljebb egy hónapig. A székletkivonatból a szabad toxin kvantitatív kimutatása céljából tizes léptékű hígítási sort készítettünk szövetkultura tápfolyadékban. A 96-lyukú microtitráló lemezben előkészített, 0,2% borjúsavót tartalmazó EAGLE-MEM médiummal fedett HeLa sejttenyészet monolayert képező sejtjeire (kb. 1000 sejt/lyuk) az előzőleg előkészített hígításokból a megfelelő lyukakba 20-20 µl-t vittünk. Pozitív kontrollként a VPI (Virginia Polytechnical Institute) *Clostridium difficile* 10.463 törzs Holman tenyészetének, a székletmintákhoz hasonlóan előkészített felülúszójának baktériummentes szűrletét használtuk (a szűrletet –20 °C-on tárolhatjuk). Negatív kontrollként a hígítóként használt szövetkultura tápfolyadék 20 µl-t mértük a lyukakba. A mintákat tartalmazó 96-lyukú lemezt 37 °C-on, 5% $CO_2$-ot tartalmazó termosztátban inkubáltuk, majd 24 óra múlva vizsgáltuk a cytotoxicus hatást a szöveten. Ha legalább a sejtek 50%-a lekerekedik, akkor + jelzést használunk, a kontrollal megegyező erősségű cytotoxicus effectust ++++-el jelöljük. A kifejezett cytotoxicus hatást mutató minták esetében a vizsgálatot antitoxinnal történő neutralisatio után megismételtük, mivel a szövettoxicitas nem specifikus a *Clostridium difficile* toxinra, más enteropathogen ágensek (vírusok, *E. coli*, *Bacteroides fragilis*, stb.) is kiválthatnak cytotoxicus hatást a különböző szövettenyészeteken. A –20 °C-on tárolt hígítatlan, baktériummentesre szűrt székletfelülúszót felengedés után 1:1 arányban (50-50 µl) elegyítettük *Clostridium difficile* antitoxint tartalmazó kecskesavóval. Az elegyet 30 percig a reakció lezajlásáig 37 °C-on inkubáltuk, majd a szövettoxicitási próbának megfelelően készült hígításokat vittük fel a szövetre párhuzamosan a nem kezelt székletkivonatokkal. A lemezt 24 órán keresztül 37 °C-on, 5% $CO_2$-ot tartalmazó termosztátban inkubáltuk, majd az inkubációs idő letelte után értékeltük. Abban az esetben, ha *Clostridium difficile* toxin jelen volt a mintában, a kecske-antitoxin semlegesítette azt, így a cytotoxicus hatás elmarad.[6]

**Clostridium difficile *toxin kimutatása VIDAS módszerrel***

Az ELISA elven alapuló módszer során a reakció egy álló fázison (pipetta hegyben) zajlik le, mely belső felszíne nyúlban termeltetett, *Clostridium difficile* A toxinnal szembeni ellenanyaggal van borítva. A mintában jelenlévő *Clostridium difficile* A toxin kötődik a specifikus antitesthez, majd a kikötődött complex a szokásos mosási eljárások után az alkalikus phosphatase-zal conjugált, egér anti-*Clostridium difficile* A toxinhoz kapcsolódik. A pipettahegy belsejében lezajló enzimreakciót egy fluorescens substrat, a methyl-umbelliferil phosphat segítségével tudjuk kimutatni. A pipettahegy belső fala katalizálja a reakciót, a végtermék a 4-metilumbelliferon fluorescencia intenzitása optikai denzitométerrel mérhető, az intenzitás arányos a mintában jelenlévő toxin mennyiségével.

A centrifugált széklet felülúszó 300 µl-nyi mennyiségét a kit első lyukába mérjük, majd a gyártó előírásainak megfelelő standardok és kontrollok beállítása után a VIDAS készülékbe helyezzük. Az eredményt 1 óra elteltével a géphez csatlakozó computer értékeli, számszerű értékelést, ill. pozitív, kétes vagy negatív minősítést ad meg. Abban az esetben, ha a megadott érték kétes a konfirmáló vizsgálatot is el kell végezni. Ebben az esetben az első lépésként 300 µl, az előzőekben ismertetett módon feldolgozott széklet felülúszót és 10 µl kecskében termeltetett anti-*Clostridium difficile* A toxin-antitestet tartalmazó "blocking" reagenst helyezünk a strip megfelelő lyukába. Egy másik strip megfelelő lyukába ugyancsak 300 µl mintát helyezünk, de ehhez a mintához 10 µl normál kecske serumot adunk. A szokásos mosási eljárások után fluorescens substrattal jelzett egér monoclonalis anti-*Clostridium difficile* A toxin-antitest kötődik a mintában jelenlévő complexhez. Abban az esetben, ha *Clostridium difficile* A toxin jelen volt a mintában, a kecske-antitoxin közömbösíti azt, így a reakció negatívvá válik, míg a másik stripben a normál kecske serum nem közömbösíti a toxint, így pozitív/kétes marad a reakció.[14] A teszt előnye, hogy a székletminta laboratóriumba érkezése után kb. 2 óra múlva már jelezheti a laboratórium az eredményt a klinikus felé.

## Eredmények

Az 1999. április 15. és decémber 15. közötti időszakban laboratóriumunkba beérkezett 1860 széklet minta döntő többsége intézetben fekvő, hospitalisált betegektől származott. A rutin feldolgozás során az összes beküldött mintából 46 esetben (2,5%) *Salmonella* speciest, 4 esetben (0,2%) *Campylobacter* speciest isoláltunk. *Yersinia* sp.-t, *Shigella* sp.-t és enteropathogen *E. coli*-t nem isoláltunk ebben az időszakban. A *Salmonella* törzseket további identifikálás, serotypisálás, valamint a járványügyi intézkedések elindítása érdekében a Csongrád megyei ÁNTSZ enteralis laboratóriumába továbbítottuk.

Ugyanebben az időszakban összesen 468 székletminta esetében végeztünk direkt *Clostridium difficile* toxin kimutatást, és 106 esetben (22,6%) tudtuk a toxin jelenlétét valamilyen módszerrel detektálni. A *Clostridium difficile* toxin kimutatására célzottan beérkezett 150 széklet közül VIDAS módszerrel 35 (23,3%) minta bizonyult toxin A pozitívnak. (Egyik székletminta esetében sem tudtunk tenyésztéssel szokványos enteralis pathogen baktériumot kimutatni.) Az általunk felállított kritériumok szerint kiválasztott 318 székletmintából 71-

ben (22,3%) mutattuk ki a *Clostridium difficile* toxin cytotoxicus hatását HeLa szövetkultúrán (*1. táblázat*). Az utóbbi esetben a minták gyűjtése és a heti egy alkalommal elvégzett szövettoxicitási próba miatt a lelet kiadására 2-7 nap múlva került sor, míg a kért vizsgálatok esetében a beérkezést követően 4-6 óra múlva értesítettük a klinikust az eredményről.

**1. táblázat. *Clostridium difficile* direkt toxin kimutatás eredménye kért és a laboratórium által indikált vizsgálat során**

| Osztály | Székletminták száma vizsgált/pozitív (%) | |
|---|---|---|
| | Célzott vizsgálat | Nem kért vizsgálat |
| Haematologia | 74/10 (14%) | 84/12 (14%) |
| Belgyógyászat | 16/6 (38%) | 93/26 (28%) |
| Urologia | 0/0 (0%) | 12/4 (33%) |
| Sebészet | 8/5 (63%) | 25/12 (48%) |
| Nőgyógyászat | 0/0 (0%) | 4/0 (0%) |
| Sebészeti ITO | 4/1 (25%) | 29/7 (24%) |
| Egyéb | 48/13 (27%) | 71/10 (14%) |
| Összesen | 150/35 (23,3%) | 318/71 (22,3%) |

A legtöbb toxin kimutatási vizsgálatot a Haematologiai Osztályokról (felnőtt/gyermek) végeztük, összesen 157 mintából 22 (14%) volt toxin pozitív (*1. táblázat*). Ezen osztályokon ugyanolyan arányban találtunk a klinikus által kért vizsgálatok között toxin pozitivitást, mint az általunk indikált vizsgálatok esetén. A Belgyógyászati Osztályon a kért 16 mintából 38%-ban találtunk toxin pozitivitást, míg a laboratórium által indított vizsgálatok esetében 93 mintából 26 esetben (28%) találtunk cytotoxicus effectust a HeLa szövetkultúrán. Ebben az időszakban egyáltalán nem gondolt *Clostridium difficile* infekcióra, így nem kért *Clostridium difficile* toxin kimutatást székletmintából az Urológiai Osztály, azonban az enteralis vizsgálatokra beérkezett székletek közül 12 minta felelt meg az általunk felállított kritériumoknak, melyből 4 (33%) bizonyult toxin pozitívnak. A Sebészeti Osztályok 8 célzott *Clostridium difficile* toxin vizsgálatot kértek, ebből 5 (65%) bizonyult pozitívnak, míg a laboratórium által indított 25 vizsgálatból 12 (48%) volt toxin pozitív. A klinikusok összesen 4 esetben kérték a *Clostridium difficile* toxin kimutatását a Sebészeti Intenzív Osztályokról, ebből 1 volt pozitív, ugyanebben az időszakban a beküldött székletmintákból 29-et választottunk ki a megfelelő kritériumok alapján, ebből 7 (24%) volt toxin pozitív. Nem kértek *Clostridium difficile* toxin vizsgálatot a Nőgyógyászati Osztályokról a vizsgálat nyolc hónapja alatt, illetve a feldolgozott 4 mintából sem találtunk toxin pozitívat. 48 minta érkezett egyéb osztályokról (gyermek, traumatológia, orthopaedia, neurológia, nephrologia, stb.) ezek 27%-a volt pozitív, míg a kiválasztott 79 mintából 10 (17%) volt pozitív.

Az összesen 107 *Clostridium difficile* toxin pozitív székletminta 107 betegtől származott. Ezek közül mindössze 54 esetében tüntette fel a kezelőorvos, hogy a beteg kapott-e antibiotikumot és milyet a minta vétele idején, vagy azt közvetlenül megelőző időszakban. A *2. táblázat* a *Clostridium difficile* széklet toxin pozitív betegek megoszlását mutatja, attól függően, hogy milyen típusú antibiotikum terápiában részesültek. Az 54 beteg közül 18 kapott kombinált antibiotikum terápiát és 36 beteg monoterápiában részesült. A legtöbb *Clostridium difficile* pozitív hasmenéses beteg beta-lactam típusú antibiotikumot kapott mint monoterápia (23 beteg) vagy mint a kombinált terápia része (16 beteg). A második leggyakoribb antibiotikum mely használatával kapcsolatban *Clostridium difficile* toxin okozta hasmenést észleltünk a quinolonok voltak. Mindössze 1-1 beteg kapott doxycyclin, vancomycin, metronidazol, illetve fluconazol terápiát a hasmenéssel egyidőben.

**2. táblázat. *Clostridium difficile* pozitív betegek megoszlása az alkalmazott antibiotikum szerint (n:54)**

| Antibiotikumok | Betegek száma |
|---|---|
| Beta-lactam antibiotikumok | 23 |
| Quinolonok | 9 |
| Doxycyclin | 1 |
| Vancomycin | 1 |
| Metronidazol | 1 |
| Fluconazol | 1 |
| Kombinált antibiotikum terápia | 18/16* |
| Összesen | 54 |

*18-ból 16 betegnél beta-lactam antibiotikum volt a kombinált terápia egyik összetevője.

## Megbeszélés

A bél normál baktériumflórája természetes ökológiai barriert (ún. colonisatiós rezisztenciát) képez a pathogen organismusokkal szemben. Hosszan tartó antibiotikum terápia vagy chemotherapia után ez a barrier sérülhet, így az endogen, vagy nosocomialisan acquirált *Clostridium difficile* toxintermelő törzsei elszaporodhatnak, és kevésbé súlyos vagy súlyos klinikai tüneteket okozhatnak. Mulligan[12] nemzetközi felmérése alapján a toxin termelő *Clostridium difficile* törzsek tünetmentes hordozása egészséges felnőttekben Európában 0,3%-os, Japánban 15%-os. George[8] vizsgálatai alapján ez az arány egészséges önkéntesekben hosszan tartó antibiotikum adás után 46%-ra növekszik. Az újszülötteknél és a mucoviscidosisban szenvedő betegeknél a *Clostridium difficile* toxin termelő törzsek asymptomaticus hordozási aránya meglehetősen magas: 50% újszülöttekben, 32%-os mucoviscidosisban.[16] *Nagy E. és mtsai.* 1986-os vizsgálatai alapján a toxintermelő törzsek tünetmentes hordozása csecsemőkorban (1 év alatti korcsoport) 25%-nak adódott, kisgyermekkorban (1-3 éves korcsoport) a hasmenéses széklet minták (ahol szokványos enteralis pathogen nem volt kimutatható) 70%-ából isoláltak toxin termelő törzset és ezek döntő többsége összefüggésbe hozható volt antibiotikum előzetes alkalmazásával.[13] A *Clostridium difficile* nosocomialis acquirálásának kockázata rendkívül magas egyes klinikai osztályokon, különösen a haematologiai, a sebészeti profilú és az intenzív osztályokon. *Johnson és mtsai.* representativ felmérése alapján járvány-mentes helyzetekben a toxin-termelő *Clostridium difficile* törzsek kórházi acquirálásának gyakorisága 21%-os.[10] A toxin-termelő *Clostridium*

*difficile* nosocomialis terjedésének rizikótényezői: a hosszan tartó hospitalisatio, magas életkor, súlyos alapbetegség(ek). *Clabots és mtsai.* vizsgálatai alapján a kórházi tartózkodás 4. hetére a betegek mintegy 50%-a "culture" pozitívvá válik.[7]

Jelen vizsgálatunk során *Clostridium difficile* toxin kimutatására a SZTE klinikáiról 1999. április 15. és november 15. között mindössze 150 kérés érkezett, melyből 35 minta (23,3%) bizonyult pozitívnak. Ugyanebben az időszakban a laboratórium további 318 olyan székletmintából végzett *Clostridium difficile* toxin kimutatást, ahol a klinikus nem gondolt toxin-termelő *Clostridium difficile* törzs előfordulására, holott a kritériumok alapján (hosszas hospitalisatio, tartós antibiotikum kezelés, hasmenés) fel kellett volna, hogy merüljön a klinikusban az esetleges toxin-termelő *Clostridium difficile* törzs kóroki szerepe. A toxin termelő törzsek előfordulási aránya megegyezett a kért és a nem kért vizsgálatok esetében (23,3 versus 22,3%), ez az adat hasonló a nemzetközi irodalomban közölt adatokhoz (~20%). Azonban amennyiben nem került volna sor a laboratóriumban a megfelelő kritériumok alapján a széklet-felülúszókból a szabad-toxin meghatározásra a klinikus által nem kért esetekben, úgy számos toxinpozitív hasmenéses esetben nem derült volna fény a *Clostridium difficile* jelenlétére és nem történtek volna meg a megfelelő járványügyi intézkedések. Felmérésünk alapján elsősorban a beta-lactam antibiotikumok és a fluoroquinolonok használata során kell gondolni a *Clostridium difficile* okozta hasmenésre.

Saját vizsgálataink is alátámasztják azt a ma még itthon nem teljes mértékben elterjedt tényt, hogy hosszabb hospitalisatio, antibiotikum fogyasztás és egyéb rizikófaktorok megléte (mint előrehaladott kor, egyéb alapbetegség) esetén, ha hasmenés lép fel elsődlegesen *Clostridium difficile* okozta hasmenésre kell gondolni. A rendelkezésre álló laboratóriumi módszerek lehetőséget biztosítanak a toxin direkt kimutatására a székletmintából, illetve felkészült laboratóriumban lehetőség van a törzs isolálására epidemiológiai vizsgálatok céljára.

## Köszönetnyilvánítás

Ez az összeállítás a Szegedi Tudományegyetem Általános Orvostudományi Kar Infekciókontroll Bizottságának felkérésére készült.

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Urbán, E., Terhes, G., Nagy, E.: **How frequently is the *Clostridium difficile* the causative agent in hospital acquired diarrhoea?**

## Summary

Toxin-producing *Clostridium difficile* is the commonest cause of nosocomial diarrhoea and, as such, poses a major problem in hospitals. The major group susceptible to disease is the elderly population. This bacterium also has a major clinical impact in the immunosuppressed host, patients undergoing surgery (especially gastrointestinal) and those with a severe underlying disease and subject to a long hospital stay. In countries where the economic conditions do not allow laboratories to carry out costly toxin detection tests on a routine basis, unless this is specifically requested by the clinician, it is difficult to evaluate the prevalence of *C. difficile*-caused diarrhoea.

In this study the frequency of *C. difficile* toxin was investigated in faecal samples from inpatients who suffer from diarrhoea during their hospital stay. The VIDAS (BioMerieux) toxin A detection kit was used if clinicians requested screening for the presence of *C. difficile* toxin. For al other diarrhoeal stool speciemens selected for screening by the laboratory, the faccal samples were suspended in PBS, the suspension was centrifuged, and the supernatant was filtered and deep-frozen until testing on the Hep2 cell line. All positive samples were neutralized with *C. difficile* antitoxin. During an 8-month period, all diarrhoeal faecal samples from both hospitals were tested for the presence of *C. difficile* toxin. Only in the case of 150 of 468 samples obtained from diarrhoeal patients did the clinicians request examination for the presence of *C. difficile* toxin. 35 (23.3%) of these samples were positive for toxin A by the VIDAS method. Of the 318 remaining faecal samples, 71, (22.3%) proved toxin-positive with the cell culture method. Surgical (52% of 33 patients), urological (33% of 12 patients) and haematological (14% of 157 patients) cases exhibited *C. difficile*-caused diarrhoea most frequently.

# IV.

# *Clostridium difficile* hasmenés két megyei kórházban

*Tusnádi Anna*, Bakró Ildikó**, Urbán Edit***, Nagy Erzsébet****


## Összefoglalás

*A szerzők azokat a kórházban fekvő hasmenős betegeket tanulmányozták, akiknél az anamnesis és a klinikai kép alapján* Clostridium difficile *hasmenés gyanúja merül fel, és akik széklet mintájából* C. difficile *toxin vizsgálat történt. A tanulmány célja az volt, hogy meghatározzák, milyen praedisponáló tényezők és milyen súlyosságú hasmenés esetén kell a klinikusnak* C. difficile *fertőzésre gondolnia. Negyven betegből nyolcnál volt a székletminta* C. difficile *toxin pozitív. A toxin — pozitív betegek többsége a betegség kialakulása előtt antibiotikumot kapott (7/8), 50 éves kor feletti volt (6/8) és hasmenésük közepesen súlyos, vagy súlyos volt (7/8). Az esetek egy harmadánál chronicus alapbetegség is jelen volt. Általában a klinikusnak azoknál a betegeknél kell a* C. difficile *hasmenés gyanúját felvetnie, akik 50 éves kornál idősebbek, akik az elmúlt két hónapban antibiotikumot kaptak, és akiknek a hasmenése közepesen súlyos, vagy súlyos. Több praedisponáló tényező egyidejű jelenléte valószínűleg nem jelent nagyobb kockázatot a betegség kialakulására.*


## Bevezetés

A *C. difficile*-t az infectiv eredetű hasmenések leggyakoribb kórokozójának tartják. A baktérium anaerob Gram-pozitív spóraképző pálca, mely legalább kétféle toxint termelhet. Két fő toxinja (A és B toxin) a legfontosabb virulentia faktorai, melyek a betegség kialakulásáért felelőssé tehetők. Azok a *C. difficile* törzsek, melyek toxint nem termelnek, megbetegedést sem okoznak. Mindkét toxin cytotoxicus hatású számos különböző sejttípusra, mindkettő fokozott vascularis permeabilitást és vérzést okoz. Az A toxin folyadék felhalmozódást vált ki a bélben, míg a B toxinnak ilyen hatása nincs. A két toxin synergicusan hat. In vitro a B toxin erősebb károsító hatást fejt ki a human colon epitheliumra, mint az A toxin.[3] Leírtak olyan *C. difficile* törzset is, mely csak B toxint termel.[8] Az infekciót általában mint antibiotikum kezeléssel összefüggő hasmenést tartják számon, azonban olyan betegeknél is kialakulhat, akik antibiotikumot nem kaptak.[1,11] A betegségre praedisponáló tényezőként szerepelhet súlyos alapbetegség (daganat, immunsuppressiv állapot, cukorbetegség, chronicus vesebetegség), cytostaticum vagy steroid kezelés, megelőző hasi műtét, hosszas kórházi ápolás, intenzív osztályon történő kezelés, szondatáplálás.[10] Leginkább az idősek — 50 éves kor felettiek — fogékonyak a fertőzésre.[4] A *C. difficile* fertőzés tünetei rendszerint az antibiotikum kezelés ideje alatt manifesztálódnak, de jelentkezhetnek az antibiotikum adás után akár két hónappal később is. Kialakulhat infekció egydózisú antibiotikum prophylaxis után is.[12]

A fertőzés tüneteinek súlyossága rendkívül széles skálán mozog, a tünetmentes hordozástól kezdve, az enyhe vagy súlyos hasmenésen át, az életet veszélyeztető colitisig. Szerencsére a súlyos infekciók fordulnak elő a legritkábban. A tünetmentes *C. difficile* hordozás 2-5-ször gyakoribb, mint maga a betegség.[7]

Magyarországon a *C. difficile* infekció bizonyítása céljából a laboratóriumok többsége nem végez rutinszerűen tenyésztést a hasmenéses betegek székletmintáiból, és a direkt toxin kimutatás lehetősége sem áll sok helyen rendelkezésre, így a betegség előfordulási gyakoriságára vonatkozó hazai adatok alig ismertek.

## Betegek és módszerek

Azokat a két megyei kórházba (Jász-Nagykun-Szolnok Megyei Hetényi Géza Kórház és Fejér Megyei Szent György Kórház) felvett betegeket tanulmányoztuk, akiknél az infektológusok az anamnesis, a klinikai kép alapján *C. difficile* hasmenés gyanúját vetették fel és *C. difficile* toxin vizsgálatot kértek a laboratótiumtól. A vizsgálati periódus 10 hónapig (1999. májusától 2000. márciusáig) tartott. A megbetegedésre hajlamosító tényezőként értékeltük, ha a beteg anamnesisében a hasmenés kezdetét megelőző két hónapban antibiotikum kezelés vagy kórházi ápolás szerepelt, továbbá az egyéb potenciális rizikófaktorokat, mint chronicus alapbetegség, műtét, szondatáplálás, intenzív osztályos kezelés, 50 év feletti életkor, szociális otthonban lakás, cytostaticum kezelés.

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A vizsgált betegek hasmenése acut kezdetű volt, legalább 2 napig tartott, a laza székletek napi száma 3 vagy több volt.

Adatokat gyűjtöttünk a következőkről: életkor, praedisponáló tényezők és azok egyidejű száma, a hasmenés súlyossága (enyhe: napi 3, közepes: 4-5, súlyos: több mint 5 híg széklet naponta). Összesítést végeztünk az alkalmazott antibiotikumról (cephalosporin, clindamycin, aminopenicillin, quinolon, többféle antibiotikum) és az alkalmazás módjáról (per os, parenteralis, mindkettő, egyik sem).

A *C. difficile* toxin kimutatásához a székletkivonatok centrifugált steril szűrletét használtuk. A széklet mintát 1:1 arányban steril physiologiás sóoldattal homogenizáltuk 2000-es fordulatszámmal centrifugáltuk, majd a felülúszót 0,45 μm-es szűrőn szűrtük. Az így nyert széklet kivonatot –20 °C-on tároltuk a vizsgálatig. A *C. difficile* A és B toxin együttes jelenlétének kimutatására a szövettoxicitási próbát használtuk. A széklet kivonat 2-es léptékű hígításait HeLa szövettenyészetre vittük. Pozitív kontrollként a VPI 10463 jelzésű toxintermelő *C. difficile* törzs BHI levestenyészetének felülúszóját használtuk. A kifejezett cytopathiás hatást mutató minták esetében a vizsgálatot specifikus *C. difficile* antitoxint tartalmazó kecske savóval történő neutralisatiót (30 perc 37 °C-on) követően értékeltük.

Minden esetben megtörténtek a rendelkezésre álló egyéb enteropathogenek (*Salmonella, Shigella, Campylobacter, Yersinia*, enteropathogen *E. coli*) kimutatására szolgáló vizsgálatok is, és pozitivitas esetén ezeket az eseteket a tanulmányból kizártuk.

## Eredmények

48 kórházban kezelt betegnél merült fel az anamnesis és a klinikai kép alapján a *C. difficile* hasmenés gyanúja. 8 beteget a vizsgálatból kizártunk, mivel egyéb enteropathogen (5 esetben *Salmonella,* 3 esetben *Campylobacter jejuni*) kórokozó szerepe igazolódott. A maradék 40 betegből 8 beteg széklete *C. difficile* toxin pozitívnak (esetek) és 32 negatívnak (kontrollok) bizonyult. A vizsgált 40 betegből 33 (82,5%) kapott antibiotikumot: 9 beteg quinolont (2 pozitív eset), 6 beteg clindamycint (2 pozitív eset), 4 beteg aminopenicillint (2 pozitív eset), 10 beteg két- vagy többféle antibiotikumot (1 pozitív eset), 4 cephalosporint (nincs pozitív eset), 7 beteg nem kapott antibiotikumot (1 pozitív eset) (*1. táblázat*). Az antibiotikumot kapók 48,5%-a oralisan, 39,4%-a parenteralisan, 12,1%-a pedig oralisan és parenteralisan is kapott gyógyszert (*2. táblázat*). Szájon át történő antibiotikum adásnál kétszer gyakrabban fordult elő *C. difficile* toxin pozitivitas (16-ból 5 esetben – 31,25%), mint parenteralis adásnál (13-ból két esetben – 15,38%). A 40 betegből 10 betegnek volt enyhe (1 pozitív eset), 16 betegnek mérsékelten súlyos (3 pozitív eset), és 14 betegnek súlyos (4 pozitív) hasmenése. A pozitív esetek 87,5%-ának a hasmenése közepesen súlyos, ill. súlyos volt.

A *C. difficile* hasmenésre hajlamosító tényezők elemzésénél azt találtuk, hogy a toxin pozitív esetek szinte kivétel nélkül antibiotikum szedők (8-ból 7 esetben) és 50 éves kor felettiek (8-ból 6 esetben) voltak. A pozitív esetek harmadánál chronicus alapbetegség is jelen volt (*3. táblázat*). Vizsgáltuk, hogy a betegeknél hány olyan kockázati tényező volt együttesen jelen, mely *C. difficile* hasmenésre hajlamosít. A pozitív esetek 87%-ánál 1-3, a megbetegedésre hajlamosító tényező volt jelen. Azt tapasztaltuk, hogy 3-nál több kockázati tényező együttes jelenléte nem jelentett nagyobb hajlamot a *C. difficile* hasmenés kialakulására.

**1. táblázat. *Clostridium difficile* hasmenés előfordulása az antibiotikum kezelés típusa szerint**

| Antibiotikum | Pozitív esetek (n = 7) | Negatív kontrollok (n = 26) |
|---|---|---|
| Aminopenicillinek | 2 | 2 |
| Cephalosporinok | – | 4 |
| Clindamycin | 2 | 4 |
| Quinolonok | 2 | 7 |
| Többféle | 1 | 9 |

**2. táblázat. *Clostridium difficile* hasmenés előfordulása az antibiotikum kezelés módja szerint**

| Antibiotikum kezelés módja | Pozitív esetek (n = 7) | Negatív kontrollok (n = 26) |
|---|---|---|
| Per os | 5 | 11 |
| Parenterális | 2 | 11 |
| Mindkettő | – | 4 |

**3.táblázat. *Clostridium difficile* hasmenésre hajlamosító tényezők megoszlása**

| Kockázati tényező | Pozitív esetek (n = 8) | Negatív kontrollok (n = 32) |
|---|---|---|
| Antibiotikum | 7 | 26 |
| 50 év feletti életkor | 6 | 19 |
| Alapbetegség | 3 | 10 |
| Műtét | 1 | 8 |
| ITO-s kezelés | – | 9 |
| Szondatáplálás | – | 7 |

## Megbeszélés

A *C. difficile* spórák szájon át jutnak a szervezetbe, megbetegedést akkor okoznak, ha a baktérium vegetativ formája a colonban meg tud telepedni és toxinképzés indul meg. A kórokozón kívül a gazdaszervezetnek is jelentős szerepe van abban, hogy kialakul-e megbetegedés, ill. hogy az milyen súlyosságú. A normál bélflóra által biztosított colonisatiós rezisztenciát tartják a legfontosabbnak a *C. difficile* fertőzéssel szembeni védelemben. Tehát minden tényező, mely a normál bélflóra összetételét megváltoztatja hajlamosíthat *C. difficile* infekcióra. Kimutatták, hogy az egészséges felnőttekben észlelt 0-

3%-os tünetmentes baktérium hordozás antibiotikum adása után átmenetileg 46%-ra emelkedik.[5,6] Az antibiotikum szedéssel összefüggő hasmenések 11-33%-ában kórokozó a *C. difficile*.[2] Az életkor előrehaladásával a tünetmentes baktérium hordozás aránya nő.[14] Jelenleg az Angliában és Walesban észlelt *C. difficile* infekciók 80%-a 65 év felettiekben fordul elő.[15] Becslések szerint egy átlagos méretű, általános feladatokat ellátó, területi kórházban a *C. difficile* fertőzések száma évente 100 körül lehet.[9,13]

Vizsgálatunk célja az volt, hogy meghatározzuk, milyen praedisponáló tényezők együttes jelenléténél és milyen súlyosságú hasmenésnél kell gondolni az infekcióra. Vizsgálatunkba csak azokat a kórházban ápolt hasmenéses betegeket vontuk be, akiknél az infektológusok az anamnesis és a klinikai kép alapján a *C. difficile* hasmenés gyanúját vetették fel, és lehetőségük volt *C. difficile* toxin vizsgálat elvégeztetésére. 48 esetből nyolcat kizártunk, mivel más enteropathogen kóroki szerepe igazolódott. A maradék 40 betegből nyolc betegnek a székletéből volt kimutatható a *C. difficile* toxin. Adataink elemzése szerint akkor kell a klinikusnak *C. difficile* fertőzésre gondolnia, ha a hasmenéses beteg 50 éves kor feletti, és ha az elmúlt két hónapban antibiotikumot kapott, a hasmenés pedig közepesen súlyos vagy súlyos.

A *C. difficile* infekció tünetei azonban széles skálán mozognak, így biztos diagnózist csak a baktérium és toxinja, vagy a direkt széklet toxin kimutatásával lehet adni. A kórházi ápolás ideje alatt kialakuló hasmenések esetén a *C. difficile* infekció gyanújának mindig fel kell merülnie, és a toxin kimutatást rutinszerűen ajánlatos lenne elvégezni azért, hogy a pozitív esetek azonosításával a megfelelő intézkedéseket meg tudjuk tenni és így elejét vehessük egy esetleges nosocomialis járvány kialakulásának.

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Tusnádi, A, Bakró I., Urbán E., Nagy E.: ***Clostridium difficile* diarrhoea in two county hospitals**


## Summary

Hospitalised patients with diarrhoea were studied whose medical histories and clinical symptoms were suspicious to *C. difficile* diarrhoea, and whose stool samples were tested for *C. difficile* toxin. The aim of the study was to assess the circumstances ( predisposing factors, severity of diarrhoea) at which the clinician should presume *C. difficile* infection. Out of forty, eight patients' faecal specimens were *C. difficile* toxin — positive. Most of the toxin — positive cases received antibiotics before the onset of the illness (7/8), were older than fifty years (6/8), and had moderate or severe diarrhoea (7/8). One third of these cases also had a chronic underlying disease. In general, the clinician should suspect *C. difficile* diarrhoea in those patients who are more than fifty, have received antibiotics in the preceding two months, and whose diarrhoea is moderate or severe. The simultaneous presence of more predisposing factors does not probably mean a greater risk for the development of the illness.

# V.

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## CORRESPONDENCE

### A clinical isolate of *Bacteroides fragilis* from Hungary with high-level resistance to imipenem

Reports from several countries have described the isolation and low prevalence of clinical strains of *Bacteroides fragilis* that carry the carbapenemase gene, *cfiA* [1–3]. In the majority of cases, the *cfiA* gene is not expressed and the isolates are sensitive to carbapenem antibiotics (MIC <8 mg/L). However, occasionally *B. fragilis* isolates are encountered (<1% of clinical strains) that show high expression of *cfiA* and high-level carbapenem resistance [1, 2]. This has been shown to result from the presence of insertion sequence (IS) element promoters, typically IS*1186* and IS*942*, immediately upstream of *cfiA*, the former being the most common [2, 4, 5].

Only sensitive strains that harbour the *cfiA* gene have been recognised in Hungary until now [3]. However, recently a highly carbapenem resistant clinical isolate of *B. fragilis* was referred to our laboratory as a part of a nationwide surveillance of antibiotic resistance among anaerobic bacteria. It was isolated from a gangrenous appendix from a 22-year-old male taken during surgery before antibiotic therapy and processed in the Department of Microbiology, University of Debrecen, Hungary. *Escherichia coli*, *Klebsiella* sp. and *Acinetobacter* sp. were also isolated. The *B. fragilis* isolate gave no zone of inhibition with a 10-$\mu$g imipenem disk, and the imipenem MIC, determined by agar dilution, was >256 mg/L; both susceptibility tests were assessed after incubation for 48 h. The specific imipenemase activity, determined by following the change of absorbance at 299 nm of a mixture of bacterial sonicate (0.2 ml), imipenem (0.2 ml, 250 mg/L) and phosphate buffer (pH 7, 0.6 ml) was 37.0 nmole of imipenem hydrolysed/min/mg of protein.

The isolate was examined by PCR to confirm the presence of the *cfiA* gene [1]. DNA upstream of the *cfiA* in the resistant strain, and the four previously isolated imipenem-sensitive Hungarian strains [3], was amplified by PCR; the primer upstream of the gene was derived from a conserved region, oligonucleotide G [4], and the downstream primer comprised the complementary sequence 565–598 within *cfiA* [6]. Gel electrophoresis revealed upstream PCR products of 1.8 kb with the resistant strain and 0.3 kb with the four sensitive strains. This suggested the insertion of an element of *c.* 1.5 kb upstream of the *cfiA* gene in the resistant strain. The presence of IS*942* (1.59 kb) was detected in the genome of the resistant strain, but not in the sensitive strains, by PCR with IS element type specific primers with sequence of nucleotides 159–186 and the complementary sequence 1419–1446 [5]. *B. fragilis* TAL3636, which possesses *cfiA* and IS*942* [5], was used as a control organism, and the PCR conditions were those described previously for *cfiA* detection [1]. The orientation and position of IS*942* relative to *cfiA* was examined by PCR mapping. The upstream and downstream primers were those used for IS*942* and *cfiA* detection, respectively, with PCR conditions similar to those employed for *cfiA* detection, except that the annealing temperature was 48°C. A PCR product of 2.2 kb was obtained, identical to that shown 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 this isolate.

This is the first time a *B. fragilis* strain has been isolated in Hungary carrying a *cfiA* gene which was highly expressed due to the presence of an efficient IS element promoter. This highlights the need to be aware of the potentially widespread distribution of these strains, and that carbapenem antibiotic sensitivity among *B. fragilis* isolates should not be taken for granted.

We thank B. Szabó, Department of Microbiology, University of Debrecen, Hungary for sending the *B. fragilis* isolates. The study visit by R. Edwards to Szeged was funded by the Royal Society and British Council.

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# VI.

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Letter to the Editor

# Isolation and characterization of an imipenem-resistant *Bacteroides fragilis* strain from a prostate abscess in a dog

*Bacteroides fragilis* group strains are the predominant constituents of the mammalian intestinal flora and are important pathogens with roles in chronic anaerobic infections. Moreover, they are the most resistant of all anaerobic bacteria to antimicrobial agents, only carbapenems and nitroimidazoles remain almost 100% active against them. Carbapenem-resistant *B. fragilis* isolates have been reported from human clinical samples in the USA, France, the UK and Japan (Cuchural et al., 1986; Podglajen et al., 1992; Edwards et al., 1999; Yamazoe et al., 1999). The carbapenem resistance mechanism is mediated by metallo-β-lactamases. Interestingly, phenotypic resistance develops only when the metallo-β-lactamase gene (*cfi*A) is preceded by an insertion-sequence (IS) element that can upregulate the expression of the resistance gene by an outward-oriented promoter (Podglajen et al., 1994, 1995). Otherwise, despite harbouring the *cfi*A gene, the isolate remains susceptible to carbapenems. Surveys on the occurrence of 'silent', but *cfi*A-positive and phenotypically resistant *B. fragilis* isolates have demonstrated their low prevalence (Podglajen et al., 1992; Edwards et al., 1999; Yamazoe et al., 1999; Sóki et al., 2000); these strains probably comprise a distinct subspecies of *B. fragilis* (Podglajen et al., 1995).

During a recent nationwide study on the prevalence of imipenem-susceptible and resistant *cfi*A-positive *B. fragilis* strains in Hungary, an isolate of *Bacteroides* from a dog was examined. This 9-year-old male German shepherd dog has a history of clinical illness of almost 4 years. Initially, at the age of 5 years, it was examined because of dysuria. The ultrasonographic picture of the prostate showed a homogeneous structure with a cystic area and hypertrophy. Routine haematological and biochemical tests were negative, except for a 92% neutrophil count and the diagnosis was diffuse subacute prostatitis. The prostatic discharge was not examined (there was no suspicion of an abscess). The symptoms persisted, despite treatment with amoxycillin/clavulanic acid and progestagens. Because of a suspected cauda equina syndrome, NSAID therapy was administered. The dog became symptom-free for some months, but a relapse then occurred. Castration was performed. At the 3-month postoperative examination, it was symptom-free; the prostate was atrophic (diameter 30 mm), with a homogeneous structure. After 3 years the symptoms reappeared. Urinalysis demonstrated haematuria and pyuria. Ultrasonography revealed a cystic area with 20 mm in diameter within

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the atrophic prostate, suggesting an abscess. Following an ultrasonographically directed puncture, therapy with oral co-trimoxazole was started, and the pus was cultured aerobically and anaerobically. The results of the cultures: *Escherichia coli* (susceptible to cefuroxim, ceftibuten, co-trimoxazole; resistant to ampicillin, amoxycillin/clavulanic acid); alpha-haemolytic *Streptococcus* (susceptible to penicillin, co-trimoxazole); *Fusobacterium nucleatum* (susceptible to penicillin, metronidazole) and *B. fragilis* (see later). In light of the above data, the treatment was supplemented with oral metronidazole and the therapy was continued for 7 weeks. Ultrasonography after 2 months indicated a shrunken abscess cavity (diameter 10 mm). The dog is currently well and its micturition is undisturbed.

The *B. fragilis* isolate was obtained on anaerobic Columbia blood agar in an anaerobic culture jar after incubation for 72 h. It was identified by means of antibiotic discs (An-Ident Discs, Oxoid, Basingstoke, UK), growth in 20% bile, indole and catalase production, esculin hydrolysis and sugar fermentation as described in the Manual of Clinical Microbiology (Jousimies-Somer et al., 1999). The antibiotic susceptibilities of the strain were determined by Etest (AB Biodisc, Solna, Sweden) after incubation at 37 °C for 48 h in an anaerobic environment (Bactron, Shell Lab., Cornelius, USA) as recommended by National Committee of Clinical Laboratory Standards (Wayne, PA). MICs for penicillin, cefoxitin, augmentin, imipenem, meropenem, clindamycine and metronidazole were >32, 128, >256, >256, >32, 1 and 4 mg/l, respectively.

This isolate was characterised to establish the resistance mechanism: the production of high-level carbapenemase activity, the presence of the *cfi*A gene and the upstream insertion of an activating IS element. The specific imipenemase activity of the isolate, determined by following the change in absorbance at 299 nm of a mixture of bacterial sonicate (0.2 ml), imipenem (0.2 ml, 250 mg/l) and phosphate buffer (pH 7, 0.6 ml) according to described previously (Edwards et al., 1999), was 37.0 nmol hydrolysed imipenem/mg protein min. The isolate harboured the *cfi*A gene, as revealed by a PCR experiment carried out as described by Edwards et al. (1999). Examination of the upstream region of the resistance gene by PCR amplification with an upstream primer derived from a conserved sequence upstream of the gene, oligonucleotide G (Podglajen et al., 1994) and a downstream primer comprising the complementary sequence 565–598 in *cfi*A (Thompson and Malamy, 1990) showed a 1.8 kb fragment, which suggested an insertion of about 1.5 kb in this region. IS type-specific PCR and PCR mapping experiments were carried out to reveal the entity of the insertion. The presence of *IS*942, which comprises 1.59 kb, was detected in the genome of this resistant isolate by using primers with the sequence of nucleotides 159–186 and the complementary sequence 1419–1446 of the *IS*942 element (Rasmussen and Kovacs, 1991). The latter two PCR experiments were carried out under the conditions described for normal *cfi*A PCR (Edwards et al., 1999), except that the elongation time in the upstream region amplification was 3 min. The position of the *IS*942 element in the genome of the resistant isolate was confirmed by PCR mapping, using the upstream and downstream primers as in *IS*942 and *cfi*A detection, respectively, with conditions similar to those for *cfi*A detection, except that the annealing temperature was 48 °C and the elongation step lasted for 3 min. A PCR product of 2.2 kb was obtained, identical to that shown by

*B. fragilis* TAL3636, which indicated close proximity and the correct orientation of *IS942* to *cfi*A to drive a high-level carbapenemase expression.

This is the first report on the isolation of an imipenem-resistant *B. fragilis* strain of domestic animal origin with the regular resistance mechanism to carbapenems, carrying a *cfi*A gene activated by the upstream insertion of an IS element. Most probably, this isolate could not have emerged from a 'silent' *cfi*A-positive bacterial cell under antibiotic selection pressure because the animal was not treated by imipenem before its isolation. However, it proves that imipenem-resistant *B. fragilis* strains are also present among domestic animals despite the fact that carbapenems are rarely used for their treatment. These strains may constitute a pool from which, besides those from normally susceptible strains, endogenous infections can arise in the animal itself but may also be transmitted to the human population.

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# VII.

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# OCCURRENCE OF METRONIDAZOLE AND IMIPENEM RESISTANCE AMONG *BACTEROIDES FRAGILIS* GROUP CLINICAL ISOLATES IN HUNGARY*

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During the period between 1987 and 1997, various surveillances of the antibiotic resistance of *B. fragilis* group isolates revealed that practically all the isolates tested were susceptible to imipenem, metronidazole and chloramphenicol; very few isolates (2.5%) exhibited resistance to amoxicillin/clavulanic acid. However, similarly as in some southern European countries, the percentages of the isolates that were resistant to ampicillin, tetracycline and clindamycin were high throughout this period, and the resistance to cefoxitin increased from 6% to 16%. In 2000, isolates with intermediate or high resistance to imipenem and isolates with increased MICs to metronidazole were emerging among the clinical isolates of *B. fragilis*. The presence of the *cfiA* gene was demonstrated by PCR in 7 of 242 isolates (2.9%); 2 of them with high MICs to carbapenems harboured the IS942 element immediately upstream of the resistance genes. In the 2 *B. fragilis* isolates with increased MICs to metronidazole, the *nim* gene could be detected by PCR. The IS1186 element was found in these isolates upregulating the metronidazole resistance gene.

*Keywords: Bacteroides* – imipenem – metronidazole – PCR – IS elements

## INTRODUCTION

In view of their high isolation rate from clinical specimens, the species of the genus *Bacteroides sensu stricto* are the most important of the anaerobic pathogens. *Bacteroides fragilis* occurs in such samples most frequently, outnumbering the related species by a factor of ten [9]. The infections, which are endogenous and often associated with facultative anaerobic species, include intra-abdominal, intra-pelvic, lung and brain abscesses, peritonitis and sepsis. The pathogenicity factors of *B. fragilis* include the capsular polysaccharide, which is a potent inducer of soft-tissue abscesses, the ability to bind to fibronectin and collagen-I, an iron uptake mechanism, lipopolysaccharide and the production of a metalloprotease toxin which causes diarrhoea [12]. Apart from their clinical significance, they are important and the most abundant ($10^{11}$ cells/g faeces) bacterial constituents of the colon, the distal small and the large bowel, where they contribute to the physiological activities of the

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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intestine, e.g. in the degradation of residual biopolymers, the biotransformation of bile acids, the production of vitamin K and the prevention of pathogenic organisms from colonization [14]. The third important feature of the genus *Bacteroides* is that its species are among the most resistant bacteria of all anaerobes and have the most numerous resistance mechanisms to antimicrobial agents [21].

Resistance against β-lactams is mediated mainly by β-lactamases, which are of two important types. The members of the first group are cephalosporinases belonging in group 2e in the Bush classification scheme [4]; they have molecular masses of 30–40 kDa, are inhibited by regular β-lactamase inhibitors such as clavulanic acid and sulbactam, hydrolyse cephalosporins rather than penicillins, and do not hydrolyse cefoxitin or carbapenems; their genes are termed *cepA* [6, 21]. The members of the second group are metallo-β-lactamases that have molecular masses of 25–33 kDa, belong in Bush functional group 3, are inhibited by EDTA, but not by regular β-lactamase inhibitors, require $Zn^{2+}$ for their activity, and are capable of hydrolysing cephamycins or carbapenems; their sequenced genes are named *cfiA* or *ccrA* [6, 21]. Clindamycin resistance in *Bacteroides* strains is mediated by a macrolide-lincomycin-streptogramin (MLS) mechanism, which causes the methylation of specific adenosine residue(s) on the 23S rRNA. The gene encoding this type of resistance, *ermF*, resides on regular transposons which may be carried on plasmids or chromosomal conjugative elements [21]. It is on these chromosomal conjugative elements, also called conjugative transposons, that the tetracycline resistance genes, *tetQ*, of *Bacteroides* are coded, which also exert ribosomal protection [21]. These conjugative transposons normally reside on the chromosome; however, as they have specific *int* and *tra* genes, they can excise from there and be transferred to another cell by a conjugative process [23]. The fourth important antibiotic resistance mechanism of *Bacteroides* strains is against nitroimidazoles; this is mediated by chromosomal or plasmid coded determinants, called *nim* genes, which are transferable by conjugation [22]. The products of these genes are nitroimidazole reductases [24]. For resistance to carbapenems and nitroimidazoles, the resistance genes should be activated by strong promoters carried on insertion sequence (IS) elements [10, 18, 24]. The *cepA* genes can be rendered more expressed by some IS elements [24], and the *ermF* genes may also be upregulated by the promoter sequences of the IS element of the regular transposon [24].

It is important to follow the changes in antibiotic resistance of *Bacteroides* and to look for genetic elements of known resistance among clinical and normal flora isolates in order to select the best treatment for the severe mixed infections they cause.

## MATERIALS AND METHODS

### *Bacteria and determination of levels of antibiotic resistance*

Bacteria belonging in the *B. fragilis* group were isolated from severe infections and were identified by conventional tests according to the guidelines of the Manual of Clinical Microbiology [11] and the Wadsworth Anaerobic Bacteriology Manual [25]

or by the ATB (bioMerieux) system. *B. fragilis* ATCC 25285 or *B. thetaiotaomicron* ATCC 29741 was used as control organism in the susceptibility tests.

Antibiotic resistance surveillances were carried out in 1987 (100 *B. fragilis* group isolates originating from one centre), in 1992 (200 *B. fragilis* group isolates from three centres), in 1994 (195 *B. fragilis* group isolates from one centre) and in 1997 (78 *B. fragilis* group isolates from six centres). The susceptibilities of these isolates were determined by the agar dilution method, by the micro-broth dilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [15] and by the newly introduced Etest (AB Biodisc, Solna, Sweden), this latter method being carried out according to the prescription of the manufacturer. MIC values were read after incubation for 48 h at 37 °C in an anaerobic chamber (Bactron, Shell Lab.).

For PCR detection of the *cfiA* and *nim* genes, 242 *B. fragilis* group isolates were selected in 1998, 1999 and 2000. The following control strains were used: *B. fragilis* TAL3636 (metallo-β-lactamase producer), *B. fragilis* NCTC9344 (carbapenem-sensitive), *B. fragilis* BF8 (*nimA*-positive) and *B. fragilis* 638R (*nim*-negative).

## *Detection of 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 [13]. One unit of β-lactamase was defined as the amount which formed 1.0 μmole of product per minute under the given conditions. The specific imipenemase activities were determined by following the change in absorbance at 299 nm of a mixture of bacterial sonicate (0.2 ml), imipenem (0.2 ml, 250 mg/l) and phosphate buffer (pH 7, 0.6 ml) and the results were given in nmole hydrolysed imipenem/mg protein/min.

## *PCR detection of imipenem and metronidazole resistance genes, and examination of their upregulating IS elements*

Template DNA was prepared by boiling of 0.5 McFarland turbidity solutions of the examined isolates for 10 min, or by the method of Wilson [28] for the *nim* gene. *CfiA* PCR reactions were performed on 5 μl of 10× PCR reaction buffer (Sigma or USB), 1 μl (2.5 mM) each of dATP, dCTP, dGTP and dTTP, 1 μl (35 pmole) each of the primers, 5 μl of DNA template and 33 μl of sterile water [7]. The primers and the amplification cycles were the same as described by Podglajen et al. [17]. In the IS element (IS1186, IS1168, IS1169, IS1170, IS4351 and IS942)-specific PCRs, we used the same reaction and amplification conditions as above, except that the sequence of the primers was as described in [10, 18, 20, 26], and annealing was for 1 min at 48 °C. In the PCR amplifying upstream region of the *cfiA* gene, the reaction and amplification conditions were the same as above, but oligonucleotide G [18] and the complementary sequence 565–598 within the *cfiA* gene (UP2) were used as

primers, the annealing was at 52 °C and the elongation lasted for 3 min. For the PCR mapping of the IS element, the upstream primer of the IS942 detection and UP2 and the *cfiA* downstream primer were used, with the same reaction and amplification conditions as above, but for elongation 3 min was applied at 72 °C.

The PCR for the *nim* gene detection was carried out as described by Trinh and Reysset [27]. The IS elements were detected as above, and the PCR mapping of the upregulating IS element was performed similarly, using the appropriate primer pair and the same reaction and amplification conditions as described for *cfiA*.

The amplification products were analysed by electrophoresis in 0.8–1.2% agarose gels containing 0.5 μg/ml ethidium bromide in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.25) at 80 V constant voltage, together with 100 bp ladder (Sigma) or the *PstI* digest of λ phage DNA as molecular weight markers, and were visualized under UV light.

## RESULTS

### *Antibiotic resistance surveillances of* B. fragilis *group isolates*

As antibiotic therapy in severe anaerobic infections is mainly empirical, it is necessary to follow the resistance levels to different antimicrobial agents of the most prevalent anaerobic pathogens from time to time. During surveillances carried out in our laboratories in 1987, 1992, 1994 and 1997, examinations were made of the resistance of *B. fragilis* group isolates to 8 antibiotics used most frequently in the empirical therapy of anaerobic infections (Table 1). Most of these studies were carried out with isolates originating from different centres in Hungary (1992, 1994 and 1997) or isolated in Szeged (1987). All isolates tested were susceptible to imipenem, metronidazole and chloramphenicol; very few isolates (2.5%) displayed resistance to amox-

*Table 1*
Prevalence of *B. fragilis* group isolates resistant to different antibiotics in Hungary in the indicated years

| Antibiotic | Percentages of resistant isolates in the following years | | | |
|---|---|---|---|---|
| | 1987 | 1992 | 1994 | 1997 |
| Ampicillin | 88 | 97 | 98 | 98 |
| Amoxicillin/clavulanic acid | n.t | 2.5 | 2.5 | 2.5 |
| Cefoxitin | 6 | 11 | 11 | 16 |
| Imipenem | n.t. | 0 | 0 | 0 |
| Tetracycline | 53 | 65 | 65 | n.t. |
| Clindamycin | 27 | 23 | 21 | 18 |
| Chloramphenicol | 0 | 0 | 0.5 | 0.5 |
| Metronidazole | 0 | 0 | 0 | 0 |

n.t. – not tested

icillin/clavulanic acid. The resistance to ampicillin and tetracycline throughout these years was high (88–98% and 53–65%, respectively). Between 1987 and 1997, the resistance to cefoxitin increased from 6% to 16%. In contrast, the clindamycin resistance of the *B. fragilis* group isolates decreased during this period, being 27% in 1987 and 18% in 1997.

## *Screening for prevalence of resistance genes among* B. fragilis *group clinical isolates*

Although no expressed resistance to carbapenems and metronidazole was detected during the resistance surveillances between 1987 and 1997, we were interested in whether the presence of the resistance genes, which could be 'silent' or expressed, can be proven among recently selected isolates.

During the period 1998–2000, a total of 242 *Bacteroides* isolates were studied by PCR for the presence of the carbapenem resistance determinant, the *cfiA* gene. Two *B. fragilis* isolates were included (isolated in 2000) which were found to be highly resistant to imipenem (MICs >256 mg/ml), both being positive for the *cfiA* gene. Five other isolates that were sensitive to imipenem (MICs <0.5 μg/ml) also harboured the *cfiA* gene (Table 2, Fig. 1).

The β-lactamase and specific imipenemase productions of the *cfiA*-positive isolates were determined (Table 2). Four of 5 isolates with low imipenem MICs produced low amounts of β-lactamases and had low MIC values, and the 2 resistant isolates produced high amounts of carbapenemase and had high imipenem and meropenem MICs. The activation mechanism of carbapenemase production was examined by looking for IS elements upstream of the *cfiA* gene in these isolates, to prove that such mechanisms are behind the upregulation of our carbapenem-resistant

*Table 2*
Carbapenem and metronidazole resistance levels, β-lactamase activities and *cfiA* and *nim* gene positivity of selected isolates

| Isolate | MIC (mg/ml) of | | | β-Lactamase activity (U/ml) | Presence of | |
|---|---|---|---|---|---|---|
| | imipenem | meropenem | metronidazole | | *cfiA* | *nim* |
| *B. fragilis* 22 | 0.06 | 0.125 | 0.125 | 0.0175 | + | – |
| *B. fragilis* 20 | 0.06 | 0.06 | 0.125 | 0.0028 | + | – |
| *B. fragilis* 98 | 0.125 | 0.25 | 0.047 | 0.0004 | + | – |
| *B. fragilis* 72 | 0.25 | 0.25 | 0.032 | 0.0109 | + | – |
| *B. fragilis* 9259/5 | >256 | >32 | 3 | 37.03* | + | – |
| *B. fragilis* 515/2 | >256 | >32 | 4 | 21.60* | + | – |
| *B. fragilis* 19924 | 0.032 | 0.047 | 12 | n.t. | – | + |
| *B. fragilis* 29877/1 | 0.125 | 1.5 | 4 | n.t. | + | + |

n.t. – not tested
*Specific imipenemase activities expresed in nmole hydrolysed imipenem/mg protein/min.

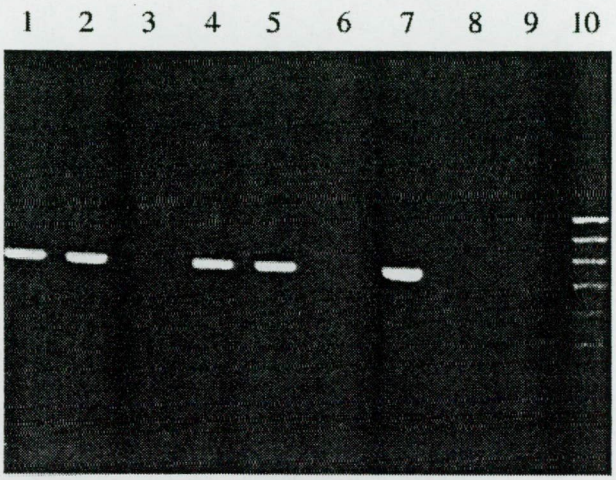
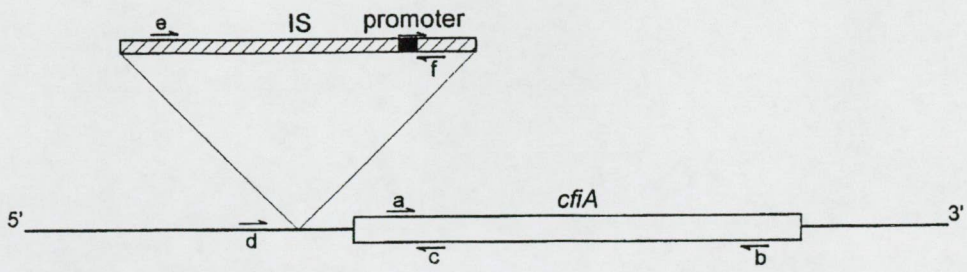



*Fig. 1. CfiA* PCR of the 'silent' *cfiA*-positive *B. fragilis* isolates. Lanes 1–8: *B. fragilis isolates* 22, 20, 92, 72, 98, 66, TAL3636 (positive control) and NCTC9344 (negative control), respectively. Lane 9: Reagent control. Lane 10: 100 bp ladder as molecular weight marker

isolates. 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), but in case of the 2 resistant isolates the ca. 1.8 kb products demonstrated the insertion of some 1.5 kb DNA segments. The presence of 5 IS elements (IS942, IS1186, IS4351, IS1169 and IS1170) in the genome of the *cfiA*-positive isolates was also investigated (Figs 2 and 3). The IS942 element was found in the 2 resistant isolates, but no IS element was detected by PCR in 4 of the 5 sensitive strains (the exception was *B. fragilis* 29877/1, which is moderately metronidazole-resistant and contains IS1186; see later). The PCR mapping of




primers:

a and b specific for *cfiA*,

c and d for the detection of an upstream insertion,

e and f specific for IS942,

e and c, b PCR mapping.

*Fig. 2.* Strategy of detection of IS elements upstream of *cfiA*

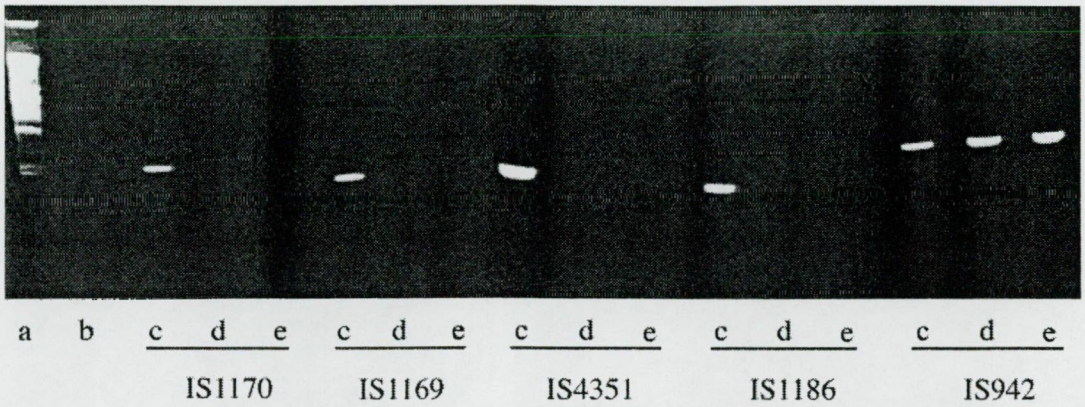



*Fig. 3.* Detection of 5 IS elements in the 2 imipenem-resistant isolates. The examination of the IS elements is indicated below the picture. a: *PstI* fragments of λ phage DNA as molecular weight marker. b: Reagent control. c: Positive controls for the indicated IS elements. d: *B. fragilis* 515/2. e: *B. fragilis* 9259/5

the IS942 elements proved that the *cfiA* genes could be upregulated by close insertion and correct orientation of the IS942 elements and by their outward-oriented promoters (Fig. 2).

Moderately metronidazole-resistant isolates could also be raised by the insertion of IS elements upstream of 'silent', chromosomally or plasmid located *nim* genes [22]. Two moderately metronidazole-resistant *B. fragilis* isolates were found in our laboratory during the first few months of 2000, with MIC values of 12 and 4 μg/ml (Table 2). One of the 2 strains carried a 'silent' *cfiA* gene as well. The *nim* PCR of these strains demonstrated a 458 bp *nim*-specific PCR product (Fig. 4). The 2 isolates were

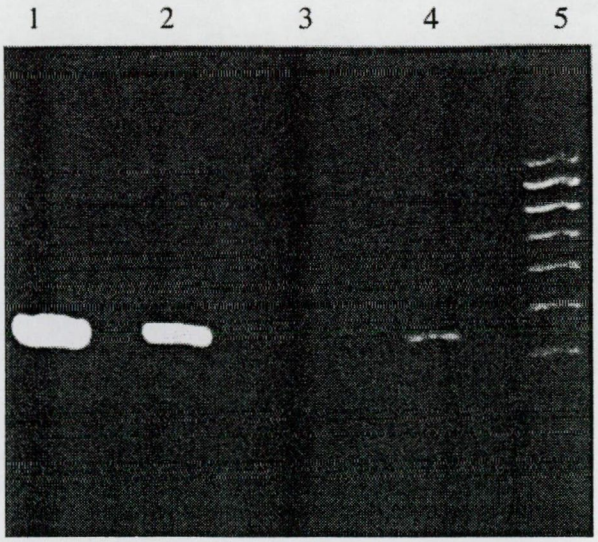



*Fig. 4. Nim* PCR of the moderately metronidazole-resistant *B. fragilis* isolates. Lanes 1–4: *B. fragilis* strains 19924, 29577, 638R (negative control) and BF8 (positive control), respectively. Lane 5: 100 bp ladder as molecular weight marker

analysed for the presence of IS elements, as in the case of the imipenem-resistant isolates. The presence of the IS1168/IS1186 element was found (these are highly homologous IS elements) in the 2 moderately resistant isolates. The PCR mapping of the IS1168 and IS1186 upstream of the *nim* gene proved that the *nim* genes of these latter isolates are activated by the close proximity and the correct orientation of one copy of the IS1168/IS1186 element. No further *nim*-positive isolates were found by PCR among the 242 metronidazole-sensitive (MICs < 2 μg/ml) *Bacteroides* isolates.

## DISCUSSION

Until several years ago, the susceptibility patterns of human pathogenic anaerobic bacteria were relatively stable and predictable. However, in some parts of the world resistance to tetracyclin, penicillin, ampicillin and erythromycin among *Bacteroides* isolates was already observed during the 1970s, and to cefoxitin and clindamycin in the 1980s. These observations and the shifting susceptibility patterns and variable efficacy of many other β-lactam antibiotics, especially the newer cephalosporins, against certain anaerobic bacteria, have made considerations concerning screening of the antibiotic susceptibilities of different groups of anaerobic bacteria mandatory [2]. Reports of differences in rates of resistance between countries and hospitals [16] revealed the great importance of monitoring trends in susceptibility from time to time. A comparison of our surveillance data with those obtained during a European study involving 15 countries showed that the *B. fragilis* group isolates in Hungary have the same resistance levels to cefoxitin and clindamycin as in Spain, a country where antibiotics are used with less control as compared with northern European countries [16]. The decrease observed in the clindamycin resistance of *B. fragilis* group strains between 1987 and 1997 demands further evaluation.

Between 1987 and 1997, similarly as in many European countries [16], no imipenem and metronidazole-resistant *Bacteroides* isolates were found in Hungary. The first imipenem-resistant *B. fragilis* isolate, producing a special metallo-β-lactamase, was reported in the USA in 1986 [5] and subsequent reports from other countries confirmed the sporadic existence [7, 21, 29]. The emergence of *Bacteroides* isolates resistant to metronidazole was first noted in France [3], and later in England [1], demonstrating that such strains may well occur elsewhere, too. It has been shown that specific events are needed for the expression of such resistances; IS elements carrying outward-oriented promoters should be inserted upstream of the 'silent' resistance genes (*cfiA* or *nim*), and the prevalence of these 'silent' genes and the activating IS elements in the population should be 'high' enough. The frequency of 'silent' and phenotypically imipenem-resistant *Bacteroides* isolates seems to be low in countries where the prevalence of the *cfiA* gene has been examined. In Hungary, the prevalence of 'silent' *cfiA*-positive and real imipenem-resistant isolates was 2.0 and 0.8%, respectively. These figures are similar to or slightly lower than the values reported in other countries: in France 1.6 and 0.8% [19], in Japan 2.4 and 1.2% [29], and in the UK 6.3 and 0.6% [7], respectively. Different IS elements were found upstream of the

*cfiA* gene. Podglajen et al. found the IS1186 insertions upstream of the *cfiA* gene [18]. Edwards et al. proved the presence of IS1187 and an element similar to the IS942/IS1170 group in meropenem-resistant strains [8]. In our *cfiA*-positive carbapenem-resistant isolates, the IS942 was confirmed in the right position upstream of the gene. The 2 metronidazole intermediate-resistant isolates harboured the IS1186/IS1168 upstream of the *nim* genes, which explains the higher MICs found in this case. No silent *nim* gene was present among our isolates, which tends to suggest the low frequency of such strains.

Special attention should be paid to the fact that clinical isolates harbouring both the *cfiA* and the *nim* gene can be found by PCR among the *B. fragilis* isolates expressing resistance and causing special therapeutic problems in severe infections.

During this study, we detected the first imipenem or metronidazole-resistant (intermediate resistance) *Bacteroides* isolates in Hungary. The increasing frequency of the presence of 'silent' genes makes it mandatory not only to continue to survey antibiotic resistance among clinical isolates of these important anaerobic pathogens, but also to screen for the presence of resistance genes among clinical and normal flora isolates.

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# VIII.

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EPIDEMIOLOGY AND BACTERIAL TYPING

# PCR ribotyping of clinically important *Clostridium difficile* strains from Hungary

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**Isolates of *Clostridium difficile* from different hospital wards at the University Hospital of Szeged in Hungary were typed by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). A total of 15 different ribotypes was detected among the 65 isolates tested. The predominant type, PCR ribotype 087, accounted for 39% of all isolates, in contrast with an international typing study where ribotype 001 was the most common. Two non-toxigenic *C. difficile* strains were found to exhibit the same pattern, which was distinct from those of all the ribotypes described previously, suggesting that this is a new type.**

## Introduction

*Clostridium difficile*, an obligately anaerobic gram-positive spore-forming rod, is a common nosocomial enteric pathogen world-wide. It causes pseudomembranous colitis (PMC), antibiotic-associated diarrhoea (AAD) or colitis (AAC) [1, 2]. The spectrum of these diseases ranges from uncomplicated mild diarrhoea to lethal toxic megacolon or colon perforation, or both [3, 4]. Since the 1980s, several studies have documented the nosocomial acquisition of *C. difficile*. Some hospital wards have a high rate of colonisation by these micro-organisms. Despite major efforts to control the spread of *C. difficile*-associated diarrhoea (CDAD) in health-care facilities, this pathogen remains a problem world-wide that continues to be responsible for both endemic and epidemic nosocomial diarrhoea [5–8]. *C. difficile* infections have become a considerable problem in most European countries, including Hungary [5–8]. 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. Typing methods have been developed on the basis of various phenotypic and genotyping markers. The phenotypic markers include toxin production, antimicrobial resistance patterns, bacteriocin and bacteriophage susceptibility patterns and whole-cell protein electrophoretic patterns, including $^{35}S$ methionine-labelled protein patterns [9–11]. Immunological markers have been described for serotyping based on the Western blot method and serogrouping by slide agglutination [12–14]. Although serotyping and phage typing are simple and useful methods, only a few laboratories can perform them because these methods require the maintenance of stocks of sera and specific phages. It has been shown that phage typing may be difficult to use because of the frequent lysogeny of *C. difficile* strains [10]. Isolates have also been fingerprinted by pyrolysis mass spectrometry (PMS), according to the profiles of the volatile products [15, 16]. Although this is a rapid technique, the disadvantage of PMS is that results for isolates that have been processed in different batches cannot be compared directly. Epidemiological markers based on genotypic characteristics are generally more discriminating and specific than phenotypic markers. Plasmid profiles, DNA restriction endonuclease and ribosomal rRNA restriction patterns have been used in the fingerprinting of *C. difficile* [17–19]. O'Neill and colleagues [20] developed a modification of the PCR ribotyping method based on polymorphisms in the 16S–32S rRNA intergenic spacer region for the routine typing of *C. difficile*. This part of the genome has been shown to be very heterogeneous, in contrast to the rRNA genes themselves, which are highly conserved. *C. difficile* was shown to possess up to 10 or more copies of the rRNA genes in its genome, which varied not only between strains, but also between different copies on the same genome. This modified PCR ribotyping

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method offers several advantages over other methods and appears to hold good promise for the typing of wild isolates of *C. difficile*.

The aim of this study was to determine the most common ribotypes of *C. difficile* in the 1200-bed University Hospital of Szeged, Hungary, and compare them with those found in the PHLS Anaerobe Reference Unit in Cardiff, UK.

## Materials and methods

### *Bacterial isolates*

Of the 65 *C. difficile* isolates investigated in this study, 57 were isolated from diarrhoeal faecal samples and 8 from other clinical materials (Table 1). 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). Isolation and identification of *C. difficile* were performed according to standard methods.

### *Toxin detection*

Single colonies were subcultured on pre-reduced Columbia Agar Base (Oxoid) supplemented with cattle blood 5%, vitamin $K_1$ and haemin and incubated at 37°C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, OR, USA) for 24 h for toxin A detection. The production of toxin A by isolates of *C. difficile* was determined by an ELISA method (ToxA TEST Immunoassay; TechLab, BioConnections, Leeds, W. Yorkshire). Toxin B was detected by cytotoxicity assay on Vero cells. The cytopathic effects of filtered chopped-meat glucose broth culture supernates (diluted 1 in 10 – 1 in $10^4$) on Vero cells were used to detect toxin B. All toxin B-positive samples were neutralised with specific *C. difficile* goat antitoxin.

### *PCR ribotyping method*

Isolates were cultured anaerobically overnight on Fastidious Anaerobe Agar (FAA; LabM, Bury) supplemented with horse blood 6% in anaerobic conditions. Approximately 10 colonies were picked and crude template nucleic acid was prepared by resuspending the cells in Chelex-100 (BioRad, Hemel Hempstead) 5% w/v and boiling for 12 min. After the removal of cellular debris by centrifugation (15 000 *g* for 10 min), 10 $\mu$l of supernate were added to 90 $\mu$l of PCR mixture containing 50 pmol of each primer, Taq polymerase (Pharmacia) 2 U and 2.25 mM $MgCl_2$. Reaction mixtures were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. Amplification products were concentrated to a final volume of 25 $\mu$l by heating at 75°C for 90 min before electrophoresis at 100 mA and 200 V in Metaphor agarose (FMC Bioproducts,

**Table 1.** Origin of isolates from the University Hospital of Szeged and their PCR ribotypes

| Origin of strains | | Number of | Number of isolates belonging to PCR ribotype | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ward | Clinical material | isolates | 001 | 002 | 009 | 010 | 012 | 015 | 032 | 040 | 068 | 070 | 087 | 092 | 114 | 124 | New type |
| Internal medicine | Faeces | 17 | 2 | | 1 | | 3 | | | 1 | 1 | | 8 | | 1 | | |
| Paediatrics | Faeces | 17 | 5 | | 1 | 1 | 3 | | 1 | | | | 4 | | | 1 | 1 |
| Surgery | Faeces | 6 | | 1 | 2 | | | | | | | 1 | 2 | | | | |
| | Drain | 3 | | | | | 1 | | | | | | 1 | | | | 1 |
| | Wound | 1 | | | | | | | | | | | 1 | | | | |
| | Bile | 1 | 1 | | | | | | | | | | | | | | |
| Haematology | Faeces | 5 | | | | | | | 1 | | | | 3 | | | 1 | |
| Dermatology | Faeces | 1 | | | | | | | | | | | 1 | | | | |
| Ophthalmology | Conjunctiva | 1 | | | | 1 | | | | | | | | | | | |
| Neurology | Faeces | 1 | | | | | | | | | | | 1 | | | | |
| Cardiac surgery | Faeces | 1 | | | | | 1 | | | | | | | | | | |
| ICU | Faeces | 9 | | | | | 4 | | | | | | 4 | 1 | | | |
| | Wound | 1 | | | | | 1 | | | | | | | | | | |
| | Intra-abdominal | 1 | | | | | | 1 | | | | | | | | | |

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

Rockland, ME, USA) 3% for 4.5 h at 8°C. Products were visualised by staining the gel for 20 min in ethidium bromide 0.5 $\mu$g/ml. To enable normalisation of all gel patterns, a molecular size standard (100 bp; Advanced Biotechnologies, Epsom) was run at five-lane intervals [20].

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

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.

## Results

The sources and PCR ribotypes of the isolates are listed in Table 1. All the isolates originated from different patients in various wards at the University Hospital of Szeged. Most of them were hospitalised in the Internal Medicine, Paediatrics and Surgery Departments and Intensive Care Unit.

In-vitro toxin testing of the isolates revealed that there were both toxigenic and non-toxigenic isolates in the samples. During a period of 3 months, 57 *C. difficile* strains were isolated from 252 faecal samples; 44 of them (77%) were toxin-producing, as detected by an immunoassay for the detection of *C. difficile* toxin A and cytotoxicity on Vero cells (toxin B). Six (75%) of the eight clinical isolates from samples other than faeces collected during the same period were toxin-producing. Toxin A-negative, toxin B-positive strains were not isolated during this period.

The PCR ribotypes consisted of patterns comprising 3–12 bands, with the size of the bands varying from *c.* 250 to 600 bp (Fig. 1). A total of 15 different ribotypes was detected among the 65 isolates tested (Table 2) [12, 21]. The 50 toxigenic isolates (77%) could be classified into seven visually distinct ribotypes, and the 15 non-toxigenic isolates into eight PCR ribotypes. Of the 50 toxigenic isolates tested, 46 (92%) belonged to three PCR ribotypes (087, 012 and 001); type 087 was the most common ribotype, accounting for 50% (25 of 50) of the toxigenic isolates tested. The remaining four isolates (8%) belonged to four other ribotypes. There was a wider distribution of ribotypes among the non-toxigenic isolates. A total of 15 toxin-negative isolates was investigated and ribotype 009 was isolated most frequently (4 of 15). Two non-toxigenic isolates belonging to ribotype 010 were isolated from conjunctiva and from the faeces of a newborn infant. This PCR ribotype is frequently isolated from the faeces of neonates and small children.

There was no significant correlation between the PCR ribotypes and the origin of the isolates investigated in this study as regards wards or patients' rooms. Some of the *C. difficile* isolates originating from different wards of the University Hospital of Szeged in Hungary belonged to the same ribotype as *C. difficile* isolates collected in the UK by the Anaerobe Reference Unit in Cardiff. All but two of the Hungarian isolates could be typed by this PCR ribotyping method, the exceptions were non-toxigenic isolates (one from faeces and one

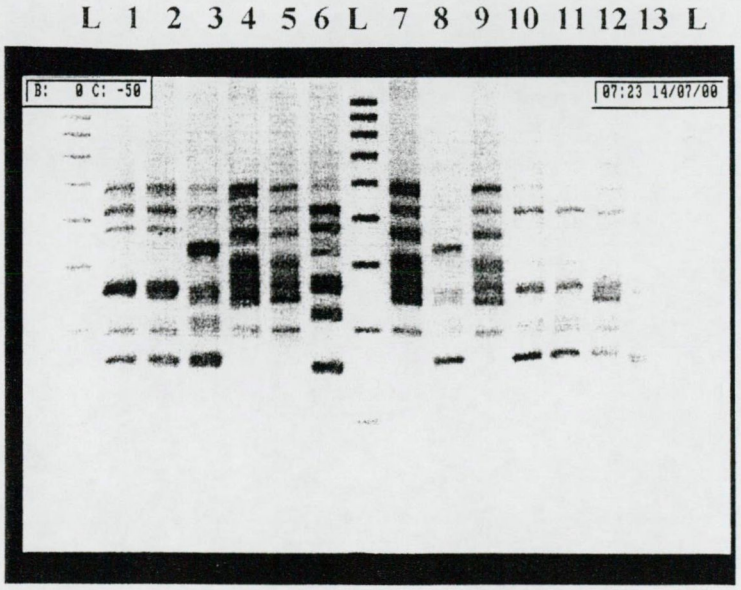


**Fig. 1.** PCR ribotype profiles found most frequently in Hungarian isolates of *C. difficile*. Lane **L**, 100-bp ladder; **1**, **2**, ribotype 015; **3**, **8**, ribotype 087; **4**, **5**, **7**, **9**, ribotype 012; **6**, ribotype 010; **10**–**13**, ribotype 001.

**Table 2.** Distribution of PCR ribotypes among Hungarian *C. difficile* isolates

| PCR ribotype | Reference strain of the ribotype* | Number (%) of isolates | Production of toxin | | Serogroup† of the reference strain | Toxino-type‡ |
|---|---|---|---|---|---|---|
| | | | A | B | | |
| 001 | R8366 | 8 (12.3) | + | + | G | 0 |
| 002 | R8375 | 1 (1.5) | + | + | A2 | 0 |
| 009 | R8269 | 4 (6.3) | – | – | I | |
| 010 | R8270 | 2 (3.0) | – | – | D,E6 | |
| 012 | R6187(S51) | 13 (20.0) | + | + | C,A,G,K | 0 |
| 015 | R6685(S38) | 1 (1.5) | + | + | G | 0 |
| 032 | R6598 | 2 (3.0) | – | – | UT | |
| 040 | R100917 | 1 (1.5) | – | – | UT | |
| 068 | IS56 | 1 (1.5) | – | – | UT | |
| 070 | R9367 | 1 (1.5) | + | + | K | I |
| 087 | R11840 | 25 (39.0) | + | + | G | 0 |
| 092 | R10630 | 1 (1.5) | + | + | UT | 0 |
| 114 | R11212 | 1 (1.5) | – | – | UT | |
| 124 | R11919 | 2 (3.0) | – | – | UT | |
| New type | – | 2 (3.0) | – | – | UT | |

UT, untypable.
*Ribotype reference strains maintained in the Anaerobe Reference Unit in Cardiff, UK.
†Serogroup of the reference strains has been made to Delmée serotype [12].
‡Toxino-type of the reference strains was determined according to methods described previously [21].

from intra-abdominal drainage) (Table 2). These two isolates exhibited the same pattern, which was distinct from those of all the ribotypes described previously, suggesting that it is a new type. More investigations are required to classify it with the cluster correlation algorithm. The integrity of the library was tested with control *C. difficile* strains: *C. difficile* NCTC 11382, ribotype 043, was used as a toxigenic control strain. The non-toxigenic control strain was *C. difficile* NCTC 11206, which belonged to PCR ribotype 038, and the control toxin A-negative, toxin B-positive strain, CCUG 20309, belonged to ribotype 036.

## Discussion

PCR ribotyping has recently been proposed as an effective means of studying *C. difficile* epidemiologically [22–24]. The PHLS Anaerobe Reference Unit in Cardiff routinely uses this method [20]. It provides a typing service for referred isolates and, from >2000 strains examined, a library consisting of 116 distinct ribotypes has been constructed. In total, 54 different PCR ribotypes have been identified from hospital patients. Type 001 accounts for 68% of the total of all isolates from hospital patients in England and Wales [25]. Type strains within the library have also been analysed by other typing schemes and the PCR ribotyping method correlates with other typing schemes and allows subtyping of many of the types produced by other methods (Table 2) [25].

The International Typing Study (involving seven groups of experts from the UK, Belgium, Australia and the USA) in 1997 organised by Brazier [26], revealed that certain types were more common in each country, indicating the distribution of the same types in hospitals in these countries. There are only a few publications about the genetic relationship of pathogenic strains of *C. difficile* from various other parts of the world, especially with regard to the Eastern European countries. In Eastern Europe there has been only one study, in a Polish maternity hospital [27], where arbitrarily primed PCR and PCR ribotyping methods were used. All environmental isolates and 11 of 31 neonatal isolates were of a single type, type 1. There have been attempts to establish some form of standardisation in the nomenclatures to decribe strains typed by the various study groups. At present these various types are uncoordinated and there is a lack of understanding as to how types relate to one another. The Polish ribotyping results have not been compared to the reference library of the Anaerobe Reference Unit in Cardiff and it could not be determined which PCR ribotype corresponds to their type 1.

The present study compared the ribotypes of 65 *C. difficile* isolates originating from patients in Szeged, Hungary, with the library of *C. difficile* ribotypes in the Anaerobe Reference Unit in the UK. Although this sample size is small, the isolates originating from Hungarian inpatients display a very different distribution of PCR ribotypes from that found by the Anaerobe Reference Unit 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, in contrast with the results of the international typing study where its prevalence was much lower. This type was not common in England and Wales either; only eight isolates of this type were found among several hundred investigated isolates [25]. The present study found two non-toxin-producing isolates, of a previously unrecognised type. No significant correlation was found between the distribution of the PCR ribotypes and the origin of the isolates during this period. Although no data have been published on the

prevalence of *C. difficile* infection or the epidemiology of *C. difficile*-associated diarrhoea in Hungary, the isolation of toxigenic *C. difficile* from hospitalised patients suggests that this pathogen may be responsible for certain cases of diarrhoea of undiagnosed origin and validates our efforts to establish its significance and conduct epidemiological studies in Hungary.

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# IX.

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# Prevalence of gastrointestinal disease caused by *Clostridium difficile* in a university hospital in Hungary

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**Summary:** A one-year survey was undertaken to investigate the frequency of diarrhoea caused by *Clostridium difficile* among patients in a 1200-bed university hospital in Hungary. The VIDAS (bioMérieux) toxin A detection kit was used for screening specimens for the presence of *C. difficile* toxin. For all other diarrhoeal specimens selected according to special criteria, cytotoxin testing was used to determine the presence of 'free toxin' in the faeces. During the study period, a total of 945 diarrhoeal faecal samples were tested for the presence of *C. difficile* toxin. Of 375 requested samples, 58 (18.3%) were toxin-A positive. Of the 570 remaining faecal samples selected by the laboratory, 120 (21%) proved to be toxin positive. The results showed that patients from the surgical (33.3%), internal (24%) and haematological (12.8%) wards had the greatest frequency of diarrhoea attributable to *C. difficile*.



*Keywords:* *C. difficile*; diarrhoea; toxin detection.

## Introduction

*Clostridium difficile* is an anaerobic spore-forming Gram-positive bacillus that produces at least two exotoxins: toxin A, an enterotoxin, and toxin B, a cytotoxin.[1,2] These are thought to be primarily responsible for the virulence of the bacterium, which is known to cause 90–100% of cases of pseudomembranous colitis,[3,4] antibiotic-associated diarrhoea and colitis.[5,6] The disease may be associated with a spectrum of severity ranging from mild diarrhoea to life-threatening and sometimes fatal pseudomembranous colitis. Rarely this may be accompanied by toxic megacolon, an electrolyte imbalance and occasional bowel perforation.[4,6–9] Toxin-producing strains are the most frequently identified cause of hospital-acquired diarrhoea. Hospital-acquired *C. difficile* in both asymptomatic and symptomatic patients, together with contamination of the hospital environment and the hands of healthcare workers, have been clearly demonstrated. The predominant risk factor is the use of antibiotics. Hospital-acquired *C. difficile* can be a serious problem for certain institutions, particularly those with high inpatient populations, chemotherapy wards, or units providing long-term patient care. It is a problem for both clinicians and the infection control team in the hospital, due to epidemic potential and cost implications.[4,10] The aims of this study were to examine the prevalence of toxin-producing *C. difficile* in faecal samples from inpatients with diarrhoea, and to evaluate physicians' requests for *C. difficile* toxin tests and their impact on the laboratory and clinical diagnoses. The results obtained on stool specimens for which *C. difficile* toxin testing was specifically

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requested were compared with those for which it was not.

## Methods

### Specimens

During a one-year period (from April 1999 to March 2000), 3081 faecal samples were screened for bacterial enteric pathogens in our laboratory, 945 of them for the presence of *C. difficile* toxin. These 945 samples, from different hospital wards at the University Hospital of Szeged, fell into two groups. The first group consisted of samples for which the clinicians had specifically requested examination for *C. difficile* toxin ($N = 375$). With these, 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 laboratory criteria for examination for the presence of 'free toxin' in the faeces using a cytotoxin assay. The selection criteria were: long-stay hospitalization (>five days), loose, liquid stools (bloody and/or mucoid), lack of other enteric pathogenic bacteria, viruses, ova or parasites, and the fact that the clinicians had not requested *C. difficile* toxin examination. The reason for using two different methods to detect the presence of *C. difficile* toxin in the two groups was that despite the rapidity of the VIDAS toxin detection kit (result in less than two hours), it was too expensive to use for the laboratory selected samples. Where previous antibiotic usage could be confirmed, the relationship between antibiotic use and the presence of diarrhoea was evaluated in the case of *C. difficile* toxin-positivity.

### Toxin testing

Freshly taken faecal samples were used for the standard cytotoxin assay using a HeLa cell line.[11] A suspension (1:1) was prepared from the faeces in phosphate-buffered saline (PBS, pH 7.4). After centrifugation (3000 **g**, 30 min), the supernatant was filtered through a 0.22 μM low protein-binding membrane filter (MILLEX®-GV) and the filtrates stored at −20°C until the *C. difficile* cytotoxin assay, which was performed once a week. Before use, the filtrates were diluted 1:10; 1:20; 1:40 and 1:80. Ten microlitres of each dilution was inoculated on to a HeLa cell monolayer, which was examined after overnight incubation and again after 48 h. *C. difficile* strain VPI 10 463 was used as positive control. To confirm the specificity, any cytopathic effect was compared with a negative PBS control and neutralization performed with *C. difficile* goat antitoxin.[12]

The automated VIDAS *C. difficile* Toxin A II (CDA2) (bioMérieux, France) system detects *C. difficile* toxin-A by using an ELFA (enzyme-linked fluorescent assay) technique. Samples that do not give a clear-cut result with this method should be tested with the confirmatory assay.[13] The advantage of this method is that the results can be reported to the clinicians within 2 h.

## Results

During the study period, 63 *Salmonella* spp. (2.05%) and four *Campylobacter* spp. isolates were found among the 3081 faecal samples (Table I), but no isolates of other enteropathogenic bacteria (*Yersinia* spp., *Shigella* spp. or enteropathogenic *Escherichia coli*) were identified.

Of the 945 stool samples investigated for the presence of *C. difficile* toxin, 178 (18.9%) were toxin-positive with one or other of the two toxin-detection methods. None of the stools positive for *C. difficile* toxin contained another enteric pathogen. These toxin-positive stool samples originated from adult inpatients (99 males, 79 females average age: 44.3 years ± 21.1). Only for 375 of the 945 stool samples obtained from diarrhoeal patients had the clinicians requested *C. difficile* toxin testing and 58 (18%) of these samples were toxin-A positive. Of the 570 remaining samples, 120 (21%) were toxin-positive by cytotoxin assay. To assess the usefulness of routine screening for *C. difficile* toxin in the faeces of long-stay diarrhoeal inpatients, the data were analysed with regard to the wards from which the samples were received (Table II).

Most samples originated from the haematological ward. The overall positivity was 13%, but 17 patients

**Table I** *Prevalence of common enteric bacterial pathogens in stool samples of inpatients (01.04.1999 and 01.04.2000)*

| Pathogens | Number of positive/tested | Percentage |
|---|---|---|
| *Salmonella* spp. | 63/3081 | 2.05 |
| *Shigella* spp. | 0/3081 | 0 |
| *Yersinia* spp. | 0/3081 | 0 |
| *Campylobacter* spp. | 4/3081 | 0.13 |
| Enteropathogenic *Escherichia coli* | 0/3081 | 0 |
| *Clostridium difficile* | 178/945* | 18.9 |

*Total includes both toxin requested and non-requested samples.

**Table II** *Incidence of* C. difficile *toxin-positivity among diarrhoeal patients from various hospital wards (01.04.1999 and 01.04.2000)*

| Hospital wards | No. of samples/No. of positives (%) | |
|---|---|---|
| | On request ($N = 375$) | After selection by the laboratory ($N = 570$) |
| Surgical | 22/7 (32%) | 50/17 (34%) |
| ICU with surgical profile | 14/2 (14%) | 46/11 (24%) |
| Internal | 50/12 (24%) | 156/37 (24%) |
| Haematological | 164/18 (11%) | 107/17 (16%) |
| Gynaecological | 2/0 (0%) | 11/2 (18%) |
| Urological | 3/0 (0%) | 19/8 (42%) |
| Others | 120/19 (16%) | 181/28 (16%) |
| Altogether | 375/58 (18%) | 570/120 (21%) |

ICU, intensive care unit.

would have been missed if only specimens submitted by the physicians specifically requesting *C. difficile* toxin testing had been examined. The highest percentage positivity was observed among patients from a surgical ward. In both groups of specimens, more than 30% proved to be toxin-positive, but the number of requested investigations was much lower than the number of specimens selected on the basis of the laboratory criteria (22 vs. 50). There were no positive tests from three samples from the urological ward for which testing had been requested, wereas 42% samples from that ward selected by the laboratory were positive. The difference in toxin-positivity rates between the two examined groups was also significant for the intensive care unit, with a surgical profile (14% positivity in the requested group versus 24% in the laboratory-selected group). Of 120 toxin-requested stool specimens submitted from other (orthopaedic, traumatological, oncological, neurological, nephrological, etc.) hospital wards, 19 (16%) were toxin-positive, while 28 (16%) of 181 non-requested specimens were also cytotoxin-positive.

It was only possible to establish which antibiotics had been prescribed and used at the time of sampling and/or one month previously for 93 patients (Table III). Of these, 37 had received antibiotic combinations and 56 had received monotherapy. Most of the patients had been treated with beta-lactam antibiotics, either alone (39 cases) or in combination with other antibiotics (29 cases). The quinolones were the second most frequently used antibiotic group showing an association with *C. difficile*-associated diarrhoea. Three patients had received tetracycline, two patients glycopeptides and

**Table III** *Distribution of* C. difficile *toxin-positive patients according to antibiotic therapy prior to diarrhoea*

| Antibiotics | No. of patients |
|---|---|
| Beta-lactam antibiotics | 39 |
| Quinolones | 10 |
| Doxycycline | 3 |
| Glycopeptides | 2 |
| Metronidazole | 2 |
| Fluconazole | 8* |
| Antibiotic combination (including beta-lactams) | 37 (29) |
| Altogether | 93 |

*Only in combination with other antibiotics.

a further two nitro-imidazoles. The antifungals administered were fluconazole (eight cases) and amphotericin B (one case), which in all cases were given in combination with other antibiotics.

## Discussion

Hospital-acquired *C. difficile* can be a serious problem for some instutions, particularly those with chemotherapy wards or units for long-term patient care. *C. difficile* is frequently acquired even in non-epidemic situations, the figure approaching 21% according to data of Johnson *et al.*[8] Risk factors associated with the acquisition of toxin-producing *C. difficile* include an advanced age, severe underlying disease, antibiotic usage and a long hospital stay. Some studies suggest that by the fourth week of hospital stay, over half of the patients are likely to be culture-positive.[14] Through the increased usage of toxin detection, it has been found that 20% diarrhoeal specimens positive for *C. difficile* or its toxins rose about four-fold in the UK between 1994 and 1997.[4,15] The role of this pathogen in hospital-acquired diarrhoea in Eastern Europe has not been clearly established. Microbiological laboratories do not carry out costly *C. difficile* toxin detection on a routine basis, only if it is specifically requested by the physician. It is therefore very difficult to evaluate the true prevalence of *C. difficile* diarrhoea in Hungarian hospitals. In our laboratory, we have found a significant number of *C. difficile* toxin-positive stools compared with the detection of other enteric pathogens, e.g., *Salmonella* sp. and *Campylobacter* sp., and these investigation were not all specifically requested. In all cases where *C. difficile* toxin was detected, the result was reported to the clinician and suitable treatment initiated. There was no significant difference in toxin-positivity rate (18% vs. 21%) between patients for whom *C. difficile*

toxin testing was specifically requested and those for whom it was not. The overall rate was 18.8%. One hundred and twenty of the 178 patients who furnished *C. difficile* toxin-positive stool specimens would have gone unnoticed if toxin testing had been carried out only when the physicians considered it necessary. Most of the toxin-positive patients were treated with beta-lactam antibiotics; the use of multiple antibiotics increases the risk of infection.[16]

This Hungarian survey should draw the attention to the role of *C. difficile* as a major hospital-acquired pathogen in patients who have undergone antimicrobial treatment, and/or surgery (especially gastrointestinal) and who are 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 examinations for it routinely.[17]

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# X.

**Short communication**

# Prevalence and characterization of *nim* genes of *Bacteroides* spp. isolated in Hungary

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Running title: *nim* gene positive *Bacteroides* isolates in Hungary

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**Abstract**

Members of the *Bacteroides* spp. are the anaerobic pathogens most commonly isolated from human clinical material. Metronidazole has been the drug of choice for the prevention and treatment of *Bacteroides* and other such anaerobic infections for nearly 40 years. Although the emergence of metronidazole-resistance in *Bacteroides* spp. was reported recently, the published rate remains low, at <5%. Isolates of *Bacteroides* spp. originating from different wards at the University Hospital of Szeged in Hungary were investigated for metronidazole resistance genes by PCR and characterized by other molecular methods. We report here the first strains of *Bacteroides* spp. with elevated MICs to metronidazole from Hungary. Of 167 isolates, 4 had metronidazole MICs >2 μg/mL, and 2 were positive for *nim* genes with metronidazole MICs of 4 and 12 μg/mL. The *nim* genes were determined to be *nimA* and *nimB,* and proved to be activated by upstream copies of the *IS1186* element. The *nim*A gene was localized in a pIP417-like plasmid, and the *nimB* gene on the chromosome.

**Keywords:** *Bacteroides fragilis,* metronidazole, *nim* genes, *IS1186,* pIP417

## Introduction

The genus *Bacteroides sensu stricto* contains 12 species of non-spore-forming, non-motile Gram-negative strict anaerobic bacilli. *Bacteroides* spp infections are mostly endogenous and often associated with facultative anaerobic and/or aerobic species; they include bloodstream infections, intra-abdominal infections, perirectal abscesses, intra-pelvic, lung and brain abscesses and soft tissue infections [1]. The choice of suitable antibiotics for therapy is limited, because the *Bacteroides* spp. are commonly resistant to many of the routinely used anti-anaerobic antibiotics [2]. Some of these resistance mechanisms may be transferable and the genetic determinants of resistance to clindamycin, erythromycin and tetracycline are well documented [3]. The 5-nitro imidazoles (5-NI), and especially metronidazole, are used worldwide in the prophylaxis and therapy of anaerobic infections due to *Bacteroides* spp. In spite of the wide use of these drugs during the last 30-40 years, only a few 5-NI-resistant strains have been isolated and only sporadic single case reports have appeared in the literature since 1978 [4-7]. Although a number of resistance mechanisms have been suggested, some metronidazole-resistant strains have been shown to possess 5-NI resistance genes, termed *nim* [8]. Five related *nim* genes have now been identified in *Bacteroides* sp. Three of them are located on low-copy number mobilizable plasmids: pIP417 (7.7 kb, *nimA*) from *B. vulgatus* BV-17, pIP419 (10 kb, *nimC*) from *B. thetaiotaomicron* BT-13, and pIP421 (7.3 kb *nimD*) from *B. fragilis* BF-F239. The fourth gene (*nimB*), which is also transferable under certain circumstances, was mapped to the chromosome of *B. fragilis* BF-8, but despite the transferable nature of the plasmids, the epidemiological data suggest that 75% of resistant isolates possess this chromosomally encoded resistance mechanism [8-10]. PCR amplification, followed by restriction enzyme analysis is a useful technique to determine the *nim* gene types, and by this means a fifth *nim* gene type, termed *nimE,* has been identified in the UK [11]. For resistance to 5-NI, the resistance genes should be activated by strong promoters carried on insertion sequence (IS) elements, and are probably 5-NI reductases capable of reducing the nitro group to an amino group without producing toxic intermediates [12].

The aim of the present study was to determine the true prevalence of the *nim* resistance genes among clinical isolates from *Bacteroides* spp. in Hungary and to characterize them.

## Materials and Methods

*Bacterial strains*

A total of 167 nonduplicate *Bacteroides* isolates were collected from the material submitted for microbiological examination from the 1200-bed University Hospital in Szeged during 2000. *Bacteroides* spp. were cultured on prereduced Columbia agar base (Oxoid, United Kingdom) supplemented with 5% cattle blood, vitamin $K_1$ and haemin, incubated at 37 °C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon, USA) for 48 hours. Routine phenotypic identification was carried out with the ATB ID 32A kit (BioMérieux, S.A., Marcy l'Etoile, France). *B. fragilis* (82) was the most commonly isolated species, constituing almost half of the total isolates submitted. *B. thetaiotaomicron* (17) was the second most common species, followed by *B. uniformis* (16), *B. ovatus* (12), *B. vulgatus* (11) *B. distasonis* (8) and *B. merdae* (7). Less commonly seen species were *B. caccae* (1), *B. eggerthii* (1) and *B. stercoris* (1). 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 blood agar.

*nim gene PCR*

The presence of *nim* genes in 4 strains that exhibited reduced sensitivity to metronidazole was assessed by PCR with primers NIM-3 and NIM-5 according to the methods described previously [10]. 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* NTCC 11295 was also investigated as a *nim* gene-negative control.

PCR products were resolved by agarose (1.5%) gel electrophoresis with a molecular weight standard (100bp; Advanced Biotechnologies, Epsom, United Kingdom), stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$) 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 with UV light after staining for 20 min with ethidium bromide (0.5 μg/mL) [12].

*Detection and mapping of activating IS elements*

The presence of the *IS1186* element was determined with the primer pairs IS1186A and IS1186B [13], using cycling parameters as follows: starting denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 sec, at 48 °C for 1 min, at 72 °C for 1 min and at 72 °C for 10 min as final elongation. The *IS1186* elements were mapped upstream of the *nimA,B* genes by using the NIM-5 and IS1186A primers, where the following cycling parameters were applied: starting denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 sec, at 50 °C for 1 min, at 72 °C for 3 min and at 72 °C for 10 min as a final elongation step. The *B. fragilis* 638R strain carrying a pIP417 plasmid was used as a positive control in both experiments. *CfiA* PCR was performed as described previously [16]. The PCR products were detected in 0.8% agarose gels by using a 100 bp ladder fragment preparation (Fermentas, Vilnius, Lithuania) as molecular weight marker, in TBE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.26) containing 0.5 μg/mL ethidium bromide and visualized as above.

*Plasmid isolation and Southern hybridization*

Plasmid DNA was isolated as described previously [14], except that chloramphenicol was not added and electrophoresed as described in the previous section. For molecular weight determination an *Escherichia coli* V517 plasmid preparation was used. DNA blotting to a Hybond $N^+$ nylon membrane (Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, UK) was carried out as in [15], *nim*A PCR fragment labelling 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. The hybridized probe was detected with the CDP-Star detection kit (Amersham Pharmacia Biotech).

**Results**

A total of 167 *Bacteroides* spp. were isolated from the various wards at the University Hospital of Szeged during a one-year period. All of them were susceptible to metronidazole (NCCLS breakpoint 16 μg/mL), but 4 *B. fragilis* isolates (2.4%) exhibited reduced sensitivity (MICs 2-12 μg/mL). These four strains were *B. fragilis* isolates 29877, 19924, 18184 and 21759 from an atherosclerotic wound, intra-abdominal pus, a diabetic foot ulcer and an appendiceal abscess, respectively. The 4 strains were all resistant to penicillin (MIC >32 μg/mL), but one strain (*B. fragilis* 29877) was multi-resistant and "silent" *cfiA*-positive [16]; all of them were susceptible to the other examined anti-anaerobic antimicrobial agents. (Table 1.) The control strains containing the four *nim* genes all yielded a PCR product of 458 bp in

length, but only 2 of the 4 examined clinical isolates gave similar PCR product. No amplification of the *nim* gene was found in the other 2 strains which displayed reduced sensitivity to metronidazole. The 4 different *nim* gene PCR products from the control strains produced unique digestion profiles with *Hpa*II and *Taq*I (Fig. 1A,B). The PCR products from *nim* genes in 2 clinical strains were identified by comparison of their digestion patterns with those of the 4 *nim* PCR fragments from the control strains. *B. fragilis* 19924 gave metronidazole MIC values of 12 μg/ml and had PCR RFLP profiles consistent with *nimA*, while *B. fragilis* 29877 had a metronidazole MIC of 4 μg/mL and had PCR RFLP profiles consistent with the *nimB* gene. In addition to the *nim* gene types, the presence of the activating IS elements was also determined. With the use of *IS1186* specific primers, the *IS1186* element was demonstrated in the genomes of both *nim*-positive strains. Using one forward primer of the *IS1186* element (IS1186A primer) and a reverse primer of the *nim* gene (NIM-5), the *IS1186* elements was proved to be located close enough upstream and in the correct orientation to the *nimA* and *nimB* genes to activate them. The plasmid profiles of the two *nim*-positive strains were also examined by a method used previously in our laboratory [15]. In the *B. fragilis* 19924 strain, a single plasmid of 7.7 kb was detected, while in the *B. fragilis* 29877 strain no plasmid could be found (Table 2). During Southern hybridization, the non-radioactively labelled *nimA* probe hybridized to the 7.7 kb plasmid forms and not to the chromosomal DNA of *B. fragilis* 19924.

**Discussion**

Metronidazole is the drug of choice for the empirical coverage of anaerobic infections. 5-NI resistance in Gram-negative anaerobes and clostridia has remained rare despite several decades of usage. The first *B. fragilis* strain resistant to metronidazole was reported by Ingham et al. in 1978 [17]. 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 rarely reported. However, the emergence of metronidazole resistance in *Bacteroides* spp. in France, [8] the UK [12], Kuwait, [18] India, [19] South Africa [20] and Morocco [21] has recently been described. The true incidence of metronidazole resistance in Hungary is probably underestimated, since the antimicrobial sensivity 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 [22]. Monitoring of the susceptibility of clinical isolates of

anaerobes, preferably by MIC determination under well-controlled anaerobic conditions, is necessary as one means of the assessing the situation. In a previous study [23], 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 intermediate resistant strains in our country. A total of 167 *Bacteroides* spp. were investigated: PCR products from *nim* genes were identified in 2 clinical strains (1.2%); one of them held *nimA* and the other the *nimB* gene, also harbouring the upstream copies of the *IS1186* element, a situation similar to that for the strains isolated and characterized previously in France [8]. 5-NI resistance genes were not detected in 2 further moderately metronidazole-resistant clinical strains (MICs 2-4 μg/mL). It is possible that other mechanisms of resistance are involved in these strains.

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 antibiotic resistance among clinical isolates of *Bacteroides* spp. and screen for the presence of *nim* resistance genes.

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Table 1. The susceptibility patterns of the four *Bacteroides fragilis* strains with reduced susceptibility to metronidazole

| Isolates | MIC (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | Penicillin | Amoxi/clav. | Imipenem | Clindamycin | Cefoxitin | Metronidazole |
| 29877 | >32 | 32 | 0.125 | 256 | 16 | 4 |
| 19924 | >32 | 1 | 0.032 | 1.5 | 1 | 12 |
| 18146 | >32 | 0.25 | 0.125 | 0.5 | 0.75 | 4 |
| 21759 | >32 | 0.25 | 0.125 | 0.032 | 1.5 | 2 |

Table 2. The properties of the *Bacteroides* spp. with reduced metronidazole susceptibility

| Species | Strain | MIC of metronidazole (μg/mL) | Type of *nim* gene | IS element | Plasmid (kb) |
|---|---|---|---|---|---|
| *B. fragilis* | 29877 | 4 | B | *IS1186* | - |
| *B. fragilis* | 19924 | 12 | A | *IS1186* | 7.7 kb |
| *B. fragilis* | 18146 | 4 | n. d. | n. t. | n. t. |
| *B. fragilis* | 21759 | 2 | n. d. | n. t. | n. t. |

n. d.: not detected

n. t.: not tested

Figure 1/A

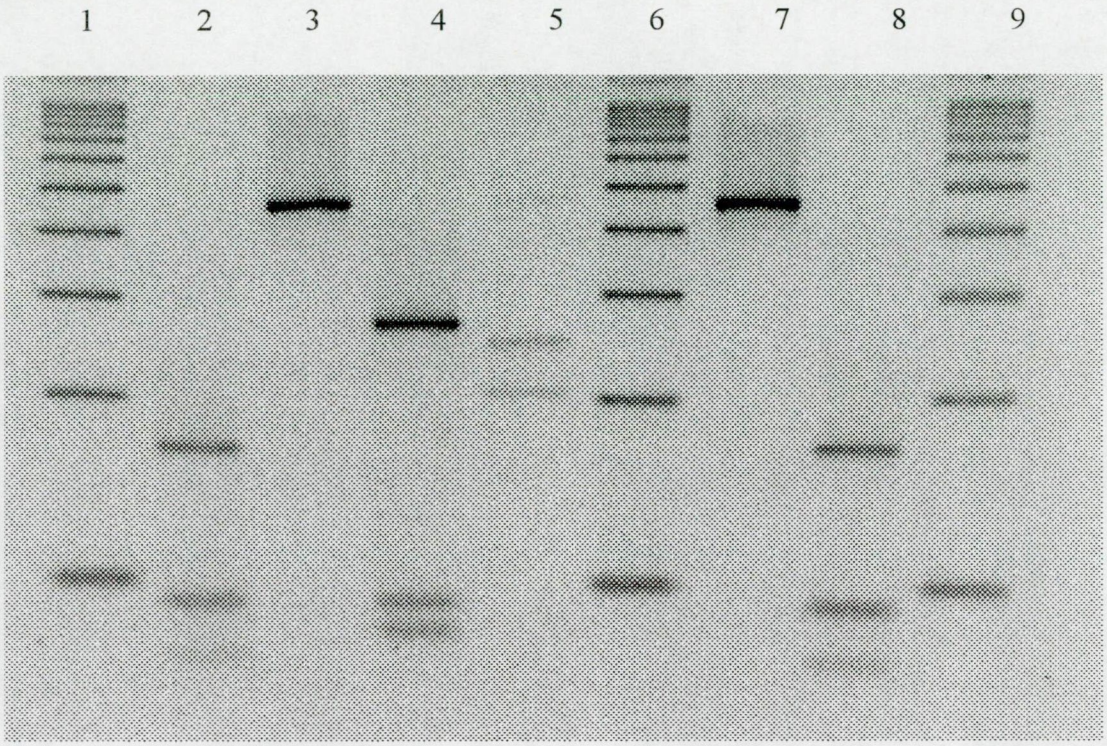
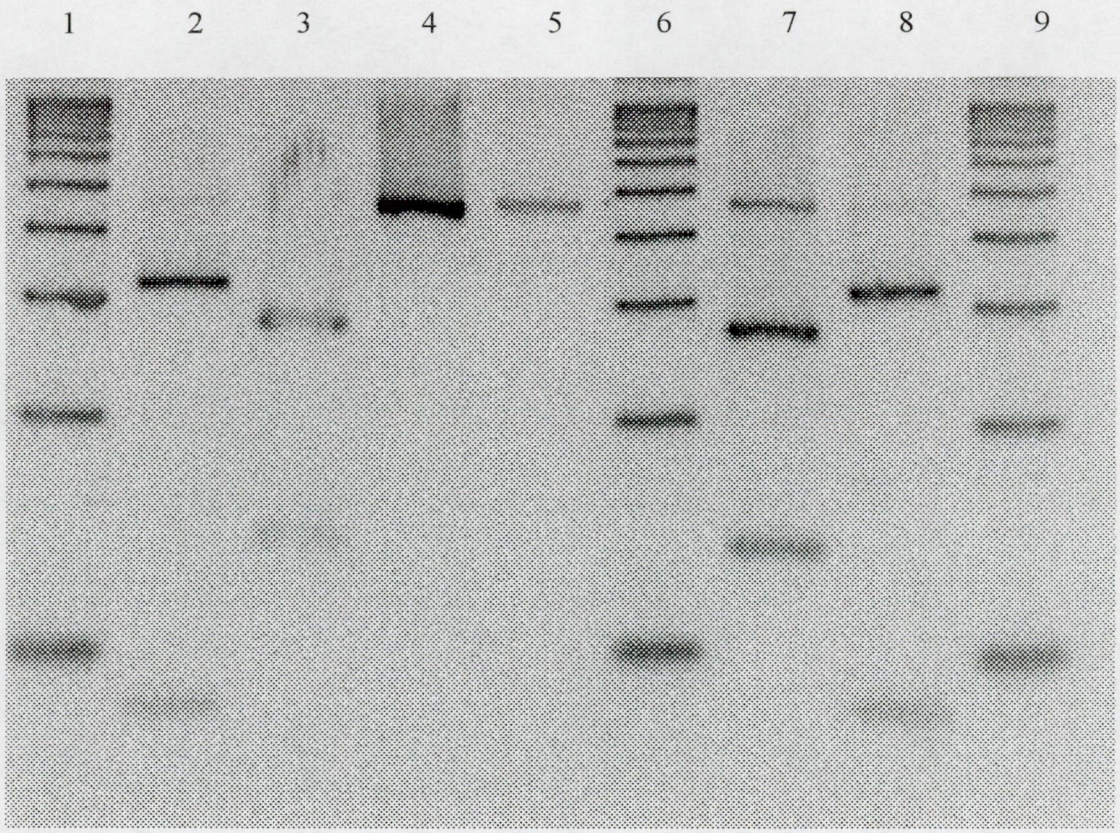



Figure 1/B



**Legends to the Figures**

Fig. 1.A RFLP profiles for *Hpa*II with genes *nim*A to *nim*D from control strains and clinical isolates

Lanes 1, 6, 9: molecular weight ladder, lanes 2-5: *nimA, nimB, nimC, nimD*- positive controls, respectively, lanes 7, 8: *B. fragilis* strains 29877/1 and 19924, respectively.

Fig 1.B. RFLP profiles for *Taq*I with genes *nim*A to *nim*D from control strains and clinical isolates

Lanes 1, 6, 9: molecular weight ladder, lanes 2-5: *nimA, nimB, nimC, nimD*-positive controls, respectively, lanes 7, 8: *B. fragilis* strains 29877/1 and 19924, respectively.