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Community-Acquired *Clostridium difficile* Diarrhea Caused by Binary Toxin, Toxin A, and Toxin B Gene-Positive Isolates in Hungary

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The aim of this work was to study the toxin types of *Clostridium difficile* isolates originating from different parts of Hungary. A PCR method was used for amplification of the two major toxin genes and the binary toxin gene and to detect the deletion or insertion in the 3′ end of the toxin A gene. The findings were compared with the results of cytotoxicity assays on the HeLa cell line. One hundred twelve isolates were tested; the toxin A and toxin B genes were detected in 79 strains by the PCR method. All of the isolates that were positive by the PCR method were also positive by the cytotoxicity assay. All of the other strains ($n = 33$) were negative for the toxin A and toxin B genes; in these cases, cytopathic effects on the cell line were not observed. No *tcdA*-negative and *tcdB*-positive isolates were found by the PCR method. In two cases, the presence of a binary toxin gene was observed by PCR; both isolates that were isolated from diarrheal feces carried the *tcdA* and *tcdB* genes. No prior hospitalization had occurred in either case.

Clostridium difficile is an anaerobic, gram-positive, spore-forming rod. Toxigenic strains are causative agents of antibiotic-associated diarrhea, antibiotic-associated colitis, and pseudomembranous colitis (1, 2). Such strains produce two major toxins: toxin A (TCDa), a cytotoxic enterotoxin that induces fluid secretion, and toxin B (TCDb), a cytotoxin. These toxins cause tissue damage in the intestinal mucosa (3). Both toxins evoke morphological changes in cell lines such as Vero, HeLa, and Hep2, but toxin B is a more potent cytotoxin than toxin A, and cytotoxicity assays therefore mainly detect toxin B.

Toxin A variant strains (*tcdA* negative, *tcdB* positive) have been reported earlier; these strains lack the repeating sequences of the *tcdA* gene. The deletion was detected by PCR because in this case the amplified fragment differs in length from the PCR product of reference strain VPI 10463. These strains are toxin A negative by commercial enzyme immunoassay, which detects only the presence of toxin A, giving a negative result, as the strains are deficient in the portion of toxin A that is immunodominant; this repeating unit is recognized by the antibodies used in the toxin A-specific detection kits. In contrast, the cytotoxicity assays are positive because of the presence of toxin B, which is an effective cytotoxin (6).

Some strains are known to produce actin-specific ADP-ribosyltransferase (CDT) (binary toxin), which is organized according to A-B toxin type. CDTa (48 kDa) is an enzymatic component, and CDTb (94 kDa) is a binding domain that recognizes the surface receptors of the host cells and mediates the cell entry of CDTa. Two subunits are encoded by separate genes: *cdtA* and *cdtB*. Actin modified by CDT induces damage to the actin cytoskeleton and leads to a cytopathic effect on the cell line (5, 9). Binary toxin-producing strains can be divided into three main groups: both toxin A and toxin B positive, both toxin A and toxin B negative, and toxin A negative and toxin B positive (10). The role of this toxin (particularly if the isolate is both toxin A and toxin B positive) in gastrointestinal infections is unclear, but the cytopathic effect on the cell line and the similarity to other clostridial binary toxins suggest that the binary toxin of *C. difficile* can be an additional virulence factor.

In Hungary, the presence of binary toxin-producing *C. difficile* strains has not been analyzed so far. Accordingly, we examined the presence of the *cdtA*, *cdtB*, *tcdA*, and *tcdB* genes, the deletion in the 3′ end of the toxin A gene, and the cytopathic effect on the HeLa cell line on *C. difficile* strains originating from fecal specimens.

Bacterial strains. One hundred twelve isolates of *C. difficile* from fecal specimens of both outpatients and inpatients were examined. These strains were isolated and identified at different laboratories in Hungary (the Budapest region, North Transdanubia, Szolnok [central Hungary], and Szeged [southern Hungary]). Forty-one strains were isolated from inpatients, 38 isolates were from outpatients, and in 33 cases we have not obtained these data yet. Reference strain VPI 10463 (toxin type 0) was obtained from the American Type Culture Collection (Rockville, Md.). Binary toxin-producing control strains were supplied by Jon Brazier (Anaerobe Reference Unit, Cardiff, United Kingdom). The strains were grown on anaerobic blood agar plates supplemented with 1 mg of vitamin K_1 per liter and 0.3 g of cysteine per liter and in brain heart infusion broth. The cultures were incubated in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2; Bactron; Sheldonb Man. Inc.) for 24 h at 37°C.

Toxin assays. The cultures were centrifuged at 15,300 × *g* for 5 min, the supernatants were filtered through a 0.22-μm-pore-size membrane (MILLEX-GV), and the filtrate was frozen immediately and kept at −20°C until used. HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal

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TABLE 1. Sequences of oligonucleotide primers used in this study[a]

Gene	Primer	Sequence (5′→3′)	Position	Size of product (bp)
cdtA	cdtApos	TGA ACC TGG AAA AGG TGA TG	507–526	375
	cdtArev	AGG ATT ATT TAC TGG ACC ATT TG	882–860	
cdtB	cdtBpos	CTT AAT GCA AGT AAA TAC TGA G	368–389	510
	cdtBrev	AAC GGA TCT CTT GCT TCA GTC	878–858	
tcdA	NK2	CCC AAT AGA AGA TTC AAT ATT AAG CTT	2479–2505	251
	NK3	GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT	2254–2283	
tcdA rep	NK9	CCA CCA GCT GCA GCC ATA	8043–8060	1,265
	NK11	TGA TGC TAA TAA TGA ATC TAA AAT GGT AAC	6795–6824	
tcdB	NK104	GTG TAG CAA TGA AAG TCC AAG TTT ACG C	2945–2972	203
	NK105	CAC TTA GCT CTT TGA TTG CTG CAC CT	3123–3148	

[a] Data from references 6, 7, and 12. rep, repetitive region.

bovine serum. Before the cytotoxicity assay, the cell culture medium was removed and fresh medium was added without serum. Tenfold dilutions of filtered bacterial culture supernatants were inoculated into the wells, and after incubation (at 37°C in 5% CO_2 for 24 h) the cytopathic effect was examined. *C. difficile* goat antitoxin serum was used for the toxin neutralization test; this antitoxin serum neutralizes both toxin A and toxin B (8).

PCR assays. A single colony of *C. difficile* was suspended in TES buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl), and the suspension was heated at 95°C for 10 min and then centrifuged at 15,300 × *g* for 5 min. The sequences of the primers used for PCR amplification in this study are listed in Table 1. NK2 and NK3 were derived from the nonrepeating sequence of the toxin A gene, while NK104 and NK105 were from the sequence of the toxin B gene. NK9 and NK11 were from the repeating sequence of the toxin A gene; these primers were used to detect the deletion of the 3′ end of the toxin A gene. The reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 μg/ml gelatin, 0.2 mM deoxynucleoside triphosphate, 0.3 U of *Taq* DNA polymerase supplied in 10 μl of Ready Mix (Sigma), and 0.15 μM cdtApos, cdtArev, cdtBpos, and cdtBrev (*cdtA* and *cdtB* PCR) or 45 ng of the NK2, NK3, NK9, NK11, NK104, and NK105 primers. The reaction mixtures were incubated for 30 (*cdtA* and *cdtB* PCR) or 35 (*tcdA* and *tcdB* PCR) cycles in a PCR system (GeneAmp, PCR System 9600; Perkin-Elmer, Norwalk, Conn.). Gene amplification consisted of denaturation at 94°C for 45 s (*cdtA* and *cdtB*) or at 95°C for 20 s (*tcdA* and *tcdB*), annealing at 52°C for 60 s (*cdtA* and *cdtB*) or at 62°C for 120 s (*tcdA* and *tcdB*), and extension at 72°C for 120 s (*cdtA* and *cdtB*) (6, 7, 12). PCR products were visualized by running in 0.8 to 1.5% agarose gels with ethidium bromide (0.5 μg/ml) at 5 V/cm in Tris-borate EDTA buffer (45 mM Tris-borate, 1 mM EDTA). Gels were photographed under UV light with a Kodak digital camera and analyzed with Kodak 1D 3.5 software.

Detection of toxin A, toxin B, and binary toxin by PCR and comparison of data obtained with the cytotoxicity assay. One hundred twelve *C. difficile* strains were examined; the presence of the toxin A and toxin B genes was observed in 79 strains, and morphological changes were demonstrated in the HeLa cell line. Thirty-three isolates were negative for both the toxin A and toxin B genes by PCR; none of those 33 isolates caused morphological damage to HeLa cells either. Eight strains had a cytotoxicity titer of 10^{-1}, 36 strains had a cytotoxicity titer of 10^{-2}, 25 strains had a cytotoxicity titer of 10^{-3}, and 10 strains had a cytotoxicity titer of $\geq 10^{-3}$. Toxin-positive isolates had no deletions or insertions in the repeating 3′ end of the toxin A gene. Two isolates were detected via the expected 375-bp (*cdtA*) and 510-bp (*cdtB*) DNA fragments by PCR with binary toxin-specific primer pairs. In both cases, no deletion could be detected in the 3′ end of the *tcdA* gene with the NK9 and NK11 primer pairs, cytopathic effects on the cell line were observed, and both the *tcdA* and *tcdB* genes were amplified by PCR from two isolates. Binary toxin-positive, toxin A- and toxin B-negative strains were not found in this study.

It is known that several *Clostridium* species may produce binary toxin; these strains generally cause gastrointestinal disease. *C. spiroforme* has been isolated from rabbit enteritis and human diarrheal feces, and animal enterotoxemia has been associated with *C. perfringens* type E (4).

The structure of the *C. difficile* binary toxin is well known, but few data are available on the role of this toxin in the pathogenesis of human gastrointestinal disease; accordingly, we collected data on the patients in whom the binary toxin-positive strains were isolated. The first binary toxin-producing strain was isolated from diarrheal feces of an 8-year-old boy. He had follicular tonsillitis and therefore received amoxicillin-clavulanic acid orally. He developed severe diarrheal disease during the therapy. The family doctor then changed the antibiotic therapy to amoxicillin, but the boy did not recover from the diarrhea. He was admitted to the gastroenterology unit of the Pal Heim Children's Hospital in Budapest. Diarrheal feces collected on the first day of his hospitalization proved positive for anaerobic, gram-positive, spore-forming rods; the colonies were irregular, with a rhizoid edge, and were identified as *C. difficile* by the RapID 32A test (BioMérieux, Marcy l'Étoile, France). Toxin A was detected by the toxin A test (Oxoid). The boy received oral metronidazole, and the alternative treatment was oral *Lactobacillus* therapy. Confirmed relapses were not observed after treatment.

The second binary toxin-producing strain was a fecal isolate from a 42-year-old woman who likewise suffered from diarrhea; the presumed diagnosis was infectious enteritis. She received oral sulfamethoxazole-trimethoprim for an upper respiratory tract infection before the appearance of her diarrhea. Gram-positive, spore-forming rods were isolated from the diarrheal feces by using CCFA (cycloserine cefoxitin fructose agar) medium and were identified as *C. difficile*, but at that time no toxin test was performed in the local laboratory. This isolate was sent to the reference laboratory (Szeged, Hungary) for toxin detection, where the presence of *tcdA*, *tcdB*, *cdtA*, and *cdtB* was detected by PCR, and cytotoxic effects on the HeLa cell line were tested. After the termination of antibiotic treatment, the condition of the patient improved without specific therapy against *C. difficile*, and no relapse was observed. In this case history too, no prior hospitalization had taken place.

In an earlier study (13), 65 *C. difficile* strains were isolated in the Szeged laboratory from patients in different wards in order to investigate the presence of toxins A and B, and ribotyping was used for epidemiological purposes in collaboration with the Anaerobe Reference Laboratory in Cardiff, United Kingdom, where the largest collection of data on different ribotypes of *C. difficile* is to be found at present. Fifteen different ribotypes were detected, including one new type with two isolates. No binary toxin-producing, toxin A-negative and toxin B-positive strains were found among these *C. difficile* isolates causing hospital-acquired diarrhea. In the present study, *tcdA*-negative and *tcdB*-positive strains were not detected by PCR among 112 isolates, but 2 strains that were isolated from community-acquired diarrhea carried the binary toxin gene. In these cases *tcdA* and *tcdB* were detected by PCR and the cytotoxicity assays were also positive. It seems that in Hungary the prevalence of binary toxin-producing strains differs considerably from the data in international studies; this discrepancy may be explained by differences in the number of isolates studied or the geographical distribution of different clones (9, 11, 12). When the number of strains studied was low, the occurrence of binary toxin-positive isolates was high (ranging from 8.3 to 23.5%); when large *C. difficile* collections were examined, the estimated prevalence was 1.6% (Japan) to 5.5% (Anaerobe Reference Unit collection) (11). Interestingly, both of the binary toxin-positive isolates observed during the present survey originated from outpatients with no previous history of hospitalization, but the pathogenic role of binary toxin could not be determined because of the presence of toxin A and toxin B. Molecular characterization of toxin variant strains is widely used, but the role of binary toxin in *C. difficile*-associated disease is still unclear. Two binary toxin-positive and toxin A- and toxin B-positive isolates suggest that this pathogen may be responsible for certain cases of community-acquired diarrhea. We therefore regard toxin detection in diarrheal feces in the community and further epidemiological examinations of Hungarian strains to be of high importance. The characterization of binary toxin-positive isolates and further patients from whom binary toxin-positive strains can be isolated may support the pathogenic role of binary toxin.

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