Prevalence and Characterization of a Binary Toxin (Actin-Specific ADP-Ribosyltransferase) from Clostridium difficile

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In addition to the two large clostridial cytotoxins (TcdA and TcdB), some strains of Clostridium difficile also produce an actin-specific ADP-ribo-syltransferase, called binary toxin CDT. We used a PCR method and Southern blotting for the detection of genes encoding the enzymatic (CDTa) and binding (CDTb) components of the binary toxin in 369 strains isolated from patients with suspected C. difficile-associated diarrhea or colitis. Twenty-two strains (a prevalence of 6%) harbored both genes. When binary toxin production was assessed by Western blotting, 19 of the 22 strains reacted with antisera against the iota toxin of C. perfringens (anti-Ia and anti-Ib). Additionally, binary toxin activity, detected by the ADP-ribosyltransferase assay, was present in only 17 of the 22 strains. Subsequently, all 22 binary toxin-positive strains were tested for the production of toxins TcdA and TcdB, toxinotyped, and characterized by serogrouping, PCR ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis. All binary toxin-positive strains also produced TcdB and/or TcdA. However, they had significant changes in the tcdA and tcdB genes and belonged to variant toxigenotypes III, IV, V, VII, IX, and XIII. We could differentiate 16 profiles by using typing methods, indicating that most of the binary toxin-positive strains were unrelated.

Clostridium difficile is a spore-forming, anaerobic, gram-positive bacillus that was first recognized in the late 1970s to be the main cause of pseudomembranous colitis (PMC) (8, 29). C. difficile is responsible for almost all cases of PMC and for 15 to 25% of cases of antibiotic-associated diarrhea (AAD) (9). In the last 20 years, C. difficile has also emerged as a major cause of nosocomial diarrhea in adult patients and has been responsible for large outbreaks in hospital settings (15, 30). For hospitalized patients, the overall incidence of C. difficile-associated diseases has been found to vary widely, from 0.1 to 2 per 100 patient admissions (13, 15, 30, 31). In many hospitals, C. difficile is the most frequently isolated enteropathogen.

Two large toxin proteins (TcdA [or toxin A] and TcdB [or toxin B]) are thought to be the primary virulence factors of C. difficile (3). These toxins are encoded by two separate genes, named tcdA and tcdB. Together with three additional genes they form a 19.6-kb pathogenicity locus called PaLoc (14, 17). TcdA and TcdB both disrupt the actin cytoskeleton of intestinal epithelial cells by the UDP-glucose-dependent glucosylation of proteins from the Rh and Ras subfamilies (26, 27).

Some strains of C. difficile also produce an actin-specific ADP-ribosyltransferase called CDT (22, 36, 42–45) which was first described by Popoff et al. in 1988 (36). The binary toxin CDT is unrelated to the well-characterized toxins TcdA and TcdB. It belongs to the group of clostridial binary toxins, which include the iota toxin of Clostridium perfringens type E, the toxin of Clostridium spiroforme, and the C2 toxin of Clostridium botulinum C and D (25, 34, 36, 37). Binary toxins consist of two independent unlinked protein chains, designated CDTa (enzymatic component) and CDTb (binding component) in C. difficile (18, 33). The binding component recognizes a cell surface receptor, resulting in the internalization of the enzymatic component into the cytosol, which catalyzes the ADP-ribosylation of monomeric actin and leads to disorganization of the cytoskeleton (1). The binary toxin locus from strain CD196 has been cloned and sequenced (33). It contains two genes (cldtA and cldtB), with an organization and sequences similar to the genes of the iota toxin of C. perfringens: the protein sequences of CDTa and CDTb are 81 and 84% similar, respectively, to the corresponding iota toxin proteins.

C. difficile and C. perfringens type E isolates produce iota toxin and have been implicated in animal enterotoxemia (11). The binary toxin is the only virulence factor identified to date for C. spiroforme, which has been implicated in rabbit enteritis and in one case of colitis in a human (2, 35). The toxigenic C. botulinum C2 toxin induces necrotic and hemorrhagic lesions of the intestine and lungs (25). In addition, C. difficile CD196, the strain in which CDT was first identified, was isolated from a patient with severe PMC (36).

Since the majority of strains isolated from symptomatic patients produce only TcdA and TcdB, one can conclude that...
CDT is not required for the virulence of *C. difficile*, but it may serve as an additional virulence factor and may function in synergy with the large clostridial cytotoxins. The aims of this study were first to determine the prevalence of *C. difficile* containing the binary toxin among strains isolated in Paris and the surrounding region and second to characterize these binary toxin-positive strains.

**MATERIALS AND METHODS**

**Bacterial strains.** We studied 369 *C. difficile* strains recovered from 288 adult patients with suspected AAD or antibiotic-associated colitis (AAC). The patients were hospitalized at 17 care facilities in Paris or the surrounding area in the year 2000. The strains were isolated from stools cultured on selective medium (tautobroth) and were cultured on selective medium (tautobroth) and then transferred to a nylon membrane (Hybond N; Pharmacia-Biotech) and probed with internal fragments of the *cdtA and cdtB* genes (353 and 490 bp, respectively) from strain CD196. The probe fragments were obtained by PCR as described above and were labeled with 32P dCTP (Boehringer, Mannheim, Germany) according to the manufacturer’s instructions.

**Detection of binary toxin CDT by Western blotting and ADP-ribosyltransferase assay.** Western blotting was performed as described by Popoff et al. (36). All strains that were positive for *cdtA and cdtB* were cultured in Wilkins-Chalgren broth for 48 h. Proteins were then precipitated from the culture supernatant with 70% ammonium sulfate (32, 36). These supernatant proteins (30 μg) were dissolved in 200 μl of distilled water, dialyzed overnight against phosphate-buffered saline, and concentrated with Acriflavine (Carlbiochem Biosciences, Inc., La Jolla, Calif.). They were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 10% acrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked by incubation for 1 h in 5% skim milk powder in phosphate-buffered saline and were incubated overnight at room temperature with rabbit polyclonal antiserum (1:5,000) specific for the enzymatic (IA) or binding (IB) component of the *C. perfringens* iota toxin. Bound antibodies were detected with peroxidase-labeled protein A and a Signal Plus kit (Pierce Chemical Co., Rockford, Ill.).

**Detection of *cdtA* and *cdtB* by Southern blot analysis.** Genomic DNAs were digested with HindIII (Ozyme, Saint Quentin en Yvelines, France), and the restriction fragments were separated by electrophoresis in a 0.8% agarose gel. They were then transferred to a nylon membrane (Hybond N; Pharmacia-Biotech) and probed with internal fragments of the *cdtA and cdtB* genes (353 and 490 bp, respectively) from strain CD196. The probe fragments were obtained by PCR as described above and were labeled with 32P dCTP (Boehringer, Mannheim, Germany) according to the manufacturer’s instructions.

**Production of TcdA and TcdB in vitro.** Strains were incubated anaerobically in brain heart infusion broth for 5 to 7 days as described by Barbut et al. (4). TcdA production was detected by use of the *P. fluorescens* TcdA assay (Meridian Diagnostics, Cincinnati, Ohio) according to the manufacturer’s instructions. TcdB was detected by a cytotoxicity assay using MRC-5 monolayers (bioMérieux, Marcy l’Etoile, France) as follows. Supernatants were filtered through a 0.45-μm-pore-size filter and 10% dilutions were applied to cells grown in 96-well plates. The characteristic cytotoxic effect (cell rounding) was observed after 18 h at 37°C (4).

**Amplification of *cdtA* and *cdtB* by PCR.** We used the primer set NK104 and NK105 from the nonrepetitive part of the *C. difficile* tcdB gene for its amplification (7). PCR was performed in 100-μl mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 0.1% Triton X-100, 1.5 mM MgCl2, and 200 μM concentration of each dNTP, 0.15 μM concentration of each primer, 1 U of Taq polymerase, and 10 μl of template DNA. PCR amplifications were performed with 30 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min 20 s.

**TABLE 1. Primers used for amplification**

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Fragment length (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td>PG48</td>
<td>CTTCTGAAACTGGGAGACTTGA</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>CCGTCAATTCTATTTAGT</td>
<td>0.353</td>
</tr>
<tr>
<td></td>
<td>Ctda pos</td>
<td>TGAACCTGAAAAGGTGATG</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Ctda rev</td>
<td>ACGATTATCTACTGGAACATTTG</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Cdtb pos</td>
<td>CTTATGGCAATGAATTACTGAG</td>
<td>0.353</td>
</tr>
<tr>
<td></td>
<td>Cdtb rev</td>
<td>ACGGGATCTCTTGCTCCATGC</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>NK104</td>
<td>GTTGTACAATGGAAGTCAAGG</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>NK105</td>
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</tr>
<tr>
<td></td>
<td>A3</td>
<td>TATTTAGCAGCTTGATATATAC</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>A4N</td>
<td>TTACAAACATATATTGCGAC</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>BI</td>
<td>AGAAATTTATGATTTGATTTATGAAAG</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>B1C</td>
<td>CAGATAAAGGAAGTAAAGTCATG</td>
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</tr>
<tr>
<td></td>
<td>B2N</td>
<td>CACCTGCACTTTAATACCTGACC</td>
<td>3.1</td>
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<td></td>
<td>APB</td>
<td>TCACGCTCGA</td>
<td>3.1</td>
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<td></td>
<td>AP4</td>
<td>GTTCGCCGCTTGACTCCCTCT</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>23S</td>
<td>CCTCGACCTTTAATACCTGACC</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* A3 and BI are PCR fragments that are amplified for toxotyping.

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amplifications were performed with 40 cycles of 95°C for 15 s, 62°C for 2 min, and 72°C for 40 s.

Toxinotyping. Toxinotyping was used to search for genetic variations in parts of the tcdA and tcdB genes. The oligonucleotide primers used were described previously by Rupnik et al. (39, 40). The first 3.1 kb of tcdB (PCR fragment B1) and a 3.7 kb fragment spanning the repetitive region of tcdA (PCR fragment A3), obtained with primer sets A3C-A4N and B1C-B2N, respectively, were screened for variations. Amplification was done for 30 cycles (fragment B1) or 35 cycles (fragment A3). PCR programs were as follows: initial denaturation for 3 min at 95°C, followed by 40 s of denaturation at 95°C, then a final extension cycle of 10 min at 72°C. The resulting amplicons were separated by electrophoresis in 0.8% gel agarose and were visualized under UV light by ethidium bromide staining. The products were also analyzed after restriction by electrophoresis in 0.8% gel agarose and were visualized under UV light by ethidium bromide staining. The products were also analyzed after restriction by electrophoresis in 0.8% gel agarose and were visualized under UV light by ethidium bromide staining.

RESULTS

Detection of tcdA and tcdB. Of the 369 strains studied (isolated from 288 patients with suspected AAD or AAC), 22 (6%) strains, isolated from 20 patients, gave amplicons of the expected sizes. These results were confirmed by Southern blotting (data not shown): a hybridization fragment was obtained with the tcdA probe and two hybridization fragments were observed with the tcdB probe, consistent with the HindIII restriction site in the tcdB probe. For each probe, all strains harbored the same hybridization profile.

The 22 positive strains corresponded to patients who were hospitalized in 11 of the 17 institutions. In the five hospitals in which >25 C. difficile strains were isolated, the prevalence of binary toxin-positive strains ranged from 0 to 13%.

Production and activity of the binary toxin. Strains with cdt genes were tested for production of the binary toxin (Table 2). On Western blots (Fig. 1), 19 of the 22 strains reacted with antisera specific to C. perfringens le. The 22 positive strains corresponded to patients who were hospitalized in 11 of the 17 institutions. In the five hospitals in which >25 C. difficile strains were isolated, the prevalence of binary toxin-positive strains ranged from 0 to 13%.

ADP-ribosyltransferase activity (Fig. 2) was detected in 17 of the 22 strains tested. Five strains (including the three giving negative results on Western blots) displayed no ADP-ribosyltransferase activity (strains 6583, 22270, 37405, 38780, and 48961) did not react with the anti-Ia and anti-Ib antisera.

ADP-ribosyltransferase activity (Fig. 2) was detected in 17 of the 22 strains tested. Five strains (including the three giving negative results on Western blots) displayed no ADP-ribosyltransferase activity (strains 6583, 22270, 37405, 38780, and 48961).
The production of TcdA and TcdB was tested only for the 22 binary toxin-positive isolates. The Premier *C. difficile* Toxin A assay was positive for all strains except strain 13376. For this strain, the A3-targeted PCR used for toxinotyping indicated a deletion of 300 bp in the repetitive domain of the *tcdA* gene.

All 22 strains gave positive results in the cytotoxicity assay for detecting TcdB. One strain (37078) had a cytotoxic effect on MRC-5 cells that differed from that of the reference strain, ATCC 43596 of serogroup C. This strain induced rounding of the cells with no spindle formation and tended to promote the formation of a discrete cluster of cells (Fig. 3).

**Toxinotyping.** All binary toxin-positive strains had changed *tcdA* and/or *tcdB* genes, and six different toxinotypes (III, IV, V, VII, IX, and XIII) were found (Table 2).

**Phenotypic and genotyping characterization of binary toxin-positive strains.** Only six strains could be typed with the antisera used. They belonged to the following serogroups: A1 (strains 20183 and 50942), G (strains 11419 and 17582), A5 (strain 50232), and C (strain 37078) (Table 2). The remaining 16 strains were nontypeable. With molecular typing methods, seven PCR ribotypes (A, B, C, D, E, F, and G) and 11 AP-PCR profiles (a to k) were identified. PFGE analysis was used to differentiate the eight strains of toxinotype V and both ribotype A and AP-PCR profile a. Three different PFGE profiles were distinguished (P1, P2, and P3). The findings for the different markers related well and showed that 16 of the 22 binary toxin-positive strains were epidemiologically unrelated.

The three patients who had strains with identical profiles (toxinotype V, PCR ribotype A, AP-PCR profile a, and PFGE profile P1) were hospitalized in three different institutions. Two pairs of strains were recovered from two patients: in one case (strains 53850 and 54953), the strains had identical profiles consistent with a relapse of infection, whereas the strains isolated from the other patient (48961 and 50942) were different by serogrouping and AP-PCR, indicating a reinfection.

**DISCUSSION**

Little is known about the clinical relevance and pathogenic role of the ADP-ribosylating toxin CDT in *C. difficile* infections. *C. difficile* induces diseases of varying severity, from mild diarrhea to PMC, toxic megacolon, and even fulminant colitis. Differences in the levels of production of toxins TcdA and TcdB alone cannot account for the wide spectrum of clinical presentations. Georges et al. (21) reported that there were no significant differences between clinical presentations according to the titer of the cytotoxin. Similarly, there was no correlation between virulence in a hamster model of AAC and the production of TcdA and TcdB in vitro (12). Therefore, the binary toxin CDT produced by some *C. difficile* strains could be responsible for these differences. CDT is a potent cytotoxin, and the damage it causes to mucosal barrier functions may prepare the way for the action of typical clostridial cytotoxins. Alternatively, CDT may act in synergy with other toxins, depolymerizing the actin cytoskeleton by a complementary mechanism. *C. difficile* strain CD196 was investigated because it causes severe PMC and it is possible that the production of this additional toxin exacerbates the symptoms of PMC (36).

In this study, we found that 22 of 369 strains (6%) isolated from 288 patients harbored binary toxin genes. An interest in binary toxin-producing strains of *C. difficile* has only developed in recent years (43), and the prevalence of binary toxin genes varies from one country to another. In 2000, a study indicated that 6.4% of the toxigenic isolates of *C. difficile* referred to the Anaerobe Reference Unit for UK hospitals possessed the *cdtA*
and cdtB genes (45). In 2003, Rupnik et al. (42) found a prevalence of 1.6% for the toxin in 310 isolates from various hospitals in Japan and Korea and from healthy infants in Indonesia. In France, Branger et al. (C. Branger, P. Ferron, L. Noussair, N. Lambert-Zikowsky, Abstr. 21st Interdisciplinary Meet. Anti-Infect. Chemother., abstr. 129/P1, 2001) reported that 9.3% of the toxigenic strains from a group of patients with diarrhea harbored the cdtA and cdtB genes. In our series of 369 strains, 214 were toxigenic (positive by tcdB PCR). The deduced prevalence of binary toxin genes in these 214 toxigenic strains (10.3%) is high, but it is similar to that obtained by Branger et al. (Abstr. 21st Interdisciplinary Meet. Anti-Infect. Chemother.). Binary toxin-producing isolates have also been isolated from patients throughout Europe and were recently reported for strains isolated in Asia, indicating that such strains are widespread (42–45). Discrepancies in the findings of prevalence reports may be partly due to the differences in patient (symptomatic or not, adult or children) or strain selection. In addition, as was shown recently by Rupnik et al., we observed that the distribution of binary toxin-positive strains can vary between hospitals (42).

The locations of the cdtA and cdtB genes in the chromosomes of C. difficile strains have not yet been reported. The identical hybridization fragments observed for all strains with either a cdtA or cdtB probe strongly suggest that our strains have a binary toxin locus inserted at the same site in the chromosome.

Only a few studies have detected the production of the CDT in strains with binary toxin genes (33, 45). The culture supernatants of most PCR-positive strains reacted with anti-Ia and anti-Ib antisera for the detection of CDTa and CDTb, but those of three strains did not. Four of the 13 strains tested by Stubbs et al. also failed to react with anti-Ia and anti-Ib antisera (45). There may be several reasons for the observed lack of reaction, including (i) differences in amino acid sequences, (ii) mutations in the gene promoters, and (iii) little or no expression of the cdtA and cdtB genes. In addition, no ADP-ribosylating activity was observed for five strains, including the three strains that were negative in Western blots. Similarly, C. difficile strains with probable silent genes for binary toxins were reported previously (33). Interestingly, two ADP-ribosyltransferase-negative strains (6583 and 38780) produced the CDTa and CDTb components. Only one similar isolate with an expressed binary toxin but with low activity was reported previously (45). Such strains could be used to identify the amino acids that are required for enzymatic activity.

Strain 37078 (toxinotype IX) had an atypical effect on MRC-5 cells which was very different from that of a reference strain (ATCC 43596). This atypical effect was, however, similar to that of TcdB of serogroup F strains (toxinotype VIII), although strain 37078 does not belong to serogroup F. The atypical effect of serogroup F strains has been shown to be similar to the effect of the lethal toxin of Clostridium sordellii (16). It has been suggested that the TcdB toxin in these strains may be a functional hybrid of the TcdB toxin of C. difficile and the lethal toxin of C. sordellii. An atypical cytotoxic effect has...
also been described for strains of toxinotype X (41), but not for strains of toxinotype IX. We are currently investigating the tcdB gene of this particular strain.

As reported by others, binary toxin genes were detected only in strains with a tcdA and/or tcdB gene that was significantly different from that of VP10463, which is the reference strain for toxinotyping (toxinotype 0) (39, 45). The only exception was reported recently by Spigaglia et al., who described one binary toxin-producing strain belonging to toxinotype 0 (44). In addition, our study confirms that toxinotype VIII is the only group of strains with significant changes in the large clostridial toxin genes that do not possess the genes for the binary toxin (45).

The characterization of strains indicated that most strains with CDT genes belonged to the rarer serogroups. Various genotypic typing methods have been employed for epidemiological studies of C. difficile. We used those that were reported to have the most discriminatory power, such as PCR ribotyping, AP-PCR, and PFGE (10). The results of the combined typing, AP-PCR, and PFGE (10). The results of the combined typing, AP-PCR, and PFGE (10).

In conclusion, we showed here that the prevalence of binary toxin genes in strains of C. difficile isolated in the Paris area is 6% and that these genes are only present in variant strains. Clinical studies are now needed to define the exact role of the binary toxin of C. difficile in human pathogenesis.

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REFERENCES


