Prevalence and pathogenicity of binary toxin–positive Clostridium difficile strains that do not produce toxins A and B

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ORIGINAL ARTICLE

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Abstract

Clostridium difficile causes antibiotic-associated diarrhoea and pseudomembranous colitis. The main virulence factors of *C. difficile* are the toxins A (TcdA) and B (TcdB). A third toxin, called binary toxin (CDT), can be detected in 17% to 23% of strains, but its role in human disease has not been clearly defined. We report six independent cases of patients with diarrhoea suspected of having *C. difficile* infection due to strains from toxinotype XI/PCR ribotype 033 or 033-like, an unusual toxinotype/PCR ribotype positive for CDT but negative for TcdA and TcdB. Four patients were considered truly infected by clinicians and were specifically treated with oral metronidazole. One of the cases was identified during a prevalence study of $A^-B^-CDT^+$ strains. In this study, we screened a French collection of 220 nontoxigenic strains and found only one (0.5%) toxinotype XI/PCR ribotype 033 or 033-like strain. The description of such strains raises the question of the role of binary toxin as a virulence factor and could have implications for laboratory diagnostics that currently rarely include testing for binary toxin.

Keywords: $A^-B^-CDT^+$ strains, binary toxin, Clostridium difficilel, diagnostic, PCR ribotype 033, toxinotype XI
Original Submission: 28 July 2014; **Revised Submission:** 15 September 2014; **Accepted:** 7 October 2014
Available online 8 November 2014

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Introduction

Clostridium difficile is responsible for 15% to 25% of antibiotic-associated diarrhoea and for more than 95% of pseudomembranous colitis. This Gram-positive spore-forming bacterium is the major cause of hospital-acquired diarrhoea [1,2]. Since 2003, many countries have reported outbreaks of severe *C. difficile* infections (CDI). This trend is assumed to be due in part to the emergence and rapid dissemination of an epidemic clone of *C. difficile* named NAP1/BI/027 [3]. Other changes have been observed in the epidemiology of CDI: (a) community cases of CDI have increased and have been described in people previously considered to be at low risk [4,5], (b) another clone, named 078, involved in severe CDI has been described [6], (c) *C. difficile* has been recognized as a pathogen or commensal in numerous animals [7,8] and (4) detection of *C. difficile* in food products has been reported [4,9].

The main virulence factors of *C. difficile* are two large clostridial toxins, toxins A (TcdA) and B (TcdB), encoded by the

http://dx.doi.org/10.1016/j.nmni.2014.10.003

genes *tcdA* and *tcdB,* respectively. These two genes are located within a locus of pathogenicity (PaLoc) with three accessory genes: *tcdC, tcdR* and *tcdE.* Sequencing of the PaLoc has indicated a series of genetic polymorphisms. On the basis of PCR–restriction fragment length polymorphism of this locus, *C. difficile* strains are currently divided into 31 toxinotypes (or toxin variant strains) that are characterized by insertions, deletions and sequence mutations compared to the reference strain, VPI 10463 (toxinotype 0) [10,11]. Among the 31 toxinotypes, only the toxinotype XI strains do not produce TcdA and TcdB (A^-B^- strains) related to an absence of the *tcdB* gene and the presence of a large deletion in the 5′ region of the *tcdA* gene [10].

A third toxin, called binary toxin or CDT, was isolated for the first time by Popoff *et al.* [12] in a patient with severe pseudomembranous colitis. This toxin is detected in 17% to 23% of *C. difficile* strains in nonoutbreak situations [13–15]. This toxin is encoded by two genes, *cdtA* and *cdtB,* located on the CDT locus (CdtLoc), which is separated from the PaLoc on the *C. difficile* chromosome [12,16]. Strains carrying the CdtLoc belonged to specific toxinotypes, e.g. toxinotypes III, IV, V and XI, or, more rarely, to strains for which the PaLoc is absent [17]. The role of binary toxin in the pathophysiology of CDI remains unclear [18].

Five nonepidemiologically related *C. difficile* strains, characterized by the absence of *tcdB* gene but the presence of the binary toxin genes, and suspected to belong to the toxinotype XI ($A^-B^-CDT^+$), were sent to the National Reference Center (NRC) for *C. difficile* in Paris, France, between December 2011 and February 2013. The objectives of this study were to confirm the toxinotype of those strains, to describe the clinical features of the five patients in whom these strains were isolated and to estimate the prevalence of such strains that produce binary toxin but not large clostridial toxins.

Materials and methods

Characterization of five strains with atypical toxin-encoding gene content received by the NRC and analysis of clinical data

Between December 2011 and February 2013, five strains with atypical toxin-encoding gene content were sent to the NRC for *C. difficile* in Paris. Three of five strains were identified as B^-CDT^+ using the molecular method Xpert® *C. difficile* (Cepheid, Sunnyvale, CA, USA) and were sent by the biologists to the NRC for confirmation of this particular result and to exclude a genetic drift in *tcdB.* The two other strains were characterized as $A^-B^-CDT^+$ by the NRC performing routine PCR targeting *tcdA, tcdB, cdtA* and *cdtB* genes on DNA extracted from *C. difficile* cultures.

DNA extraction from colonies of *C. difficile* was performed with the InstaGene Matrix kit® (Bio-Rad Laboratories, Hercules, CA, USA). Amplification by PCR of *tcdA, tcdB, tcdC, cdtA* and *cdtB* genes coding for toxin A, toxin B, TcdC and the binary toxin, respectively, was performed using primers described elsewhere [13,19]. The toxinotypes were determined according to the method described by Rupnik *et al.* [19]. Briefly, amplification of A2 and A3 fragments of *tcdA* and B1, B2 and B3 fragments of *tcdB* was performed as described. Fragment A3 was digested using *Eco*RI to determine the toxinotype. PCR ribotyping was performed as described by Bidet *et al.* [20].

In vitro toxin production was tested by inoculating two to five colonies into brain–heart infusion broth (Oxoid, Hampshire, England, UK) that was incubated 5 days under an anaerobic atmosphere. The supernatant was filtered (0.22 µm pore size), and the filtrate was inoculated on MRC-5 cells. Toxin detection was also tested by *C. diff* Quik Chek complete® (Alere, Orlando, FL, USA) performed directly on colonies. Clinical data were reviewed and analyzed for the five patients harbouring these strains.

Screening of a A^-B^- strain collection

To estimate the frequency of $A^-B^-CDT^+$ strains, 220 consecutive nontoxinogenic strains (A^-B^-) isolated in Paris (North of France) (n = 84) and Montpellier (South of France) (n = 136) between July 2011 and April 2013 were screened. All strains were isolated from patients with diarrhoea who were suspected of having CDI, who were admitted in Saint Antoine (Paris) or Arnaud de Villeneuve (Montpellier) hospitals and strains were characterized as nontoxinogenic strains by toxigenic culture (TC). The *in vitro* determination of *C. difficile* isolates ability to produce toxins (TC) was performed either by inoculating supernatant from a 5-day brain–heart infusion broth (Oxoid) on MRC-5 cells (Paris) or by using ImmunoCard® Toxins A&B (Meridian Bioscience, Cincinnati, OH, USA) directly on colonies as recommended by the manufacturer (Montpellier).

Absence of the PaLoc was confirmed as described elsewhere by obtaining an amplification of about 700 bp using primers lok1 and lok3 anchored outside the targeting PaLoc [21]. In case of a negative result (*i.e.,* absence of the PaLoc was not confirmed), amplification by PCR of *tcdA, tcdB, tcdC, cdtA* and *cdtB* genes, toxinotyping and PCR ribotyping were performed as described above [13].

Results

Characteristics of five $A^-B^-CDT^+$ strains and associated clinical data

Amplification of B1, B2 and B3 fragments of *tcdB* was confirmed as negative for the five strains (data not shown). Only the 3′

region (A2 and A3 fragments) of the *tcdA* gene was amplified for all the strains. Binary toxin genes and a 39 bp deletion in *tcdC* were identified in the five isolates. They were confirmed to be toxinotype XI strains after enzymatic restriction. Three isolates were defined as toxinotype XIa (*Eco*RI restriction pattern of A3 PCR fragment was of type 5d) and two isolates as XIb (*Eco*RI restriction pattern of A3 PCR fragment was of type 8) (Fig. 1). The five strains showed either a similar or slightly different PCR ribotyping banding pattern as reference strains (R11402, 542 and CD219), suggesting that the five strains belonged to the same lineage (Fig. 2). They were characterized as PCR ribotype 033 (or 033-like) isolates. The strains tested negative for their *in vitro* toxin production either by the cytotoxicity assay on MRC-5 cells or the *C. diff* Quik Chek complete® assay.

Clinical and biological data of the five patients were analyzed (Table 1). The five patients were hospitalized in French hospitals in different areas, and their infections were considered to be epidemiologically unrelated. All five patients were symptomatic (diarrheic stools) and had risk factors for CDI (antibiotics n = 5, age >65 years n = 3). Four of five patients harbouring toxinotype XI strains were considered truly infected by their treating physician and were specifically treated with oral metronidazole. The clinical presentation of one patient was atypical because diarrhoea was probably the manifestation of ileal CDI, as the patient had previously undergone a total colectomy (Table 1, patient 1). One patient was not treated for CDI, but symptomatic treatment was started and diarrhoea resolved in the following days (patient 3).

Prevalence of $A^-B^-CDT^+$ strains

Among the 220 studied strains, an amplification was obtained with primers lok1 and lok3, confirming the absence of the PaLoc, for 219 strains (99.5%). No amplification could be obtained for *tcdA* and *tcdB*. The binary toxin genes were also absent. These 219 strains were confirmed as nontoxigenic strains ($A^-B^-CDT^-$). Absence of amplification with primers lok1 and lok3 was observed for one strain (0.5%). Further amplifications for *tcdA* and *tcdB* showed the presence of the 3′ region of *tcdA* only. This strain harboured a 39 bp deletion in the *tcdC* gene. Binary toxin genes were present, and the strain was characterized as toxinotype XIb. This strain belonged to the same PCR ribotype (033/033-like) as the five toxinotype XI strains previously identified in our laboratory (Fig. 2). Clinical data for the patient hospitalized in Hôpital Saint Antoine, Paris, and harbouring this strain were sparse; no specific treatment was initiated despite clinical symptoms, probably because the strain was reported as nontoxigenic by TC to the physician (Table 1, patient 6).

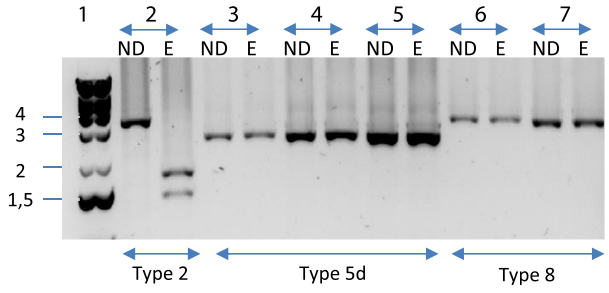

FIG. 1. Restriction patterns obtained for A3 amplified fragment of *tcdA* gene for *Clostridium difficile* $A^-B^-CDT^+$ strains isolated from patients compared with reference strains. ND, unrestricted; E., *Eco*RI digestion. Lanes: 1, DNA ladder (kb); 2, PCR ribotype 027; 3, 542 (reference strain for toxinotype XIa); 4, CD219 (reference strain for toxinotype XIa); 5, strain isolated from patient 1; 6, R11402 (reference strain for toxinotype XIb); 7, strain isolated from patient 2.

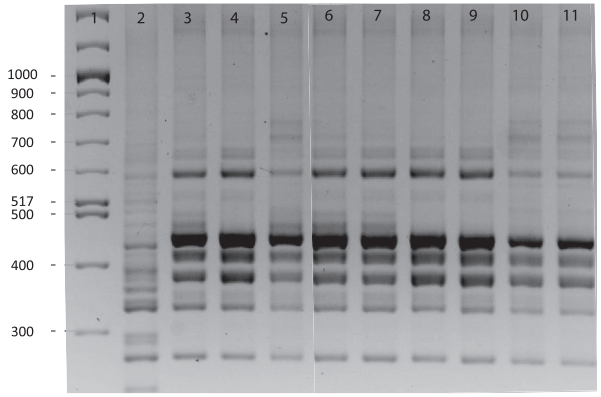

FIG. 2. PCR ribotype of *Clostridium difficile* $A^-B^-CDT^+$ strains isolated from patients compared with reference strains. Lanes: 1, DNA ladder (bp); 2, PCR ribotype 027; 3, R11402 (reference strain; toxinotype XIb); 4, patient 2; 5, patient 4; 6, strain isolated in prevalence study; 7, 542 (reference strain; toxinotype XIa); 8, patient 1; 9, CD219 (reference strain; PCR ribotype 033; toxinotype XIa); 10, patient 3; 11, patient 5.

Discussion

We report here the six first strains of toxinotype XI isolated in patients suspected of having CDI in France. Toxinotype XI strains produce only binary toxin but not the common large clostridial toxins TcdA and TcdB. Toxinotype XI strains are atypical because they exhibit major rearrangement of the PaLoc. Only portion of the 3′ end region of PaLoc is present, bearing part of the sequence of *tcdA* and *tcdC*, and the 5′ region covering *tcdB*, *tcdR* and *tcdE* is deleted [10]. Rupnik *et al.* [22] described heterogeneity in the A3 region of *tcdA* that leads to distinguish two subtypes, XIa and XIb. So far, toxinotype XI is

TABLE 1. Clinical and biological data of the six patients harbouring $A^-B^-CDT^+$ strains

Patient no.	Diagnostic	Toxino-type	Date of admission (dd/mm/yy)	Sampling date (dd/mm/yy)	Age (years)	Gender	Ward/ hospital	Location (city)	Main reason for admission	White blood cell count	Origin of diarrhoea	Type of diarrhoea	*Salmonella, Shigella, Campylo-bacter, Yersinia*	Previous antibiotics	Specific treatment for CDI	Outcome
1	B^-CDT^+ (Xpert *C. difficile* Assay, Cepheid)	XIa	02/12/2011	29/12/2011	70	Male	Vascular surgery/ Hôpital Henri Mondor	Créteil (North of France)	Surgical site infection after aortobifemoral prosthetic bypass	7.8×10^9/L	HC-CDI	Watery	Absent	Imipenem, gentamicin	MTZ po, 500 mg 3 times a day	Diarrhoea resolved/ no recurrence
2	B^-CDT^+ (Xpert *C. difficile* Assay, Cepheid)	XIb	05/10/2012	07/10/2012	81	Female	Emergency and internal medicine, infectious disease/ Hôpital André Mignot	Versailles (North of France)	Pneumonia	2.9×10^9/L	HC-CDI	Watery and mucoid	Absent	Piperacillin and tazobactam	MTZ po 500 mg 3 times a day	Diarrhoea resolved/ no recurrence
3	$A^-B^-CDT^+$ (NRC, PCR on colonies)	XIa	27/11/2012	03/12/2012	89	Male	Long-term care/ Hôpital de Bourg-en-Bresse	Bourg-en-Bresse (Centre of France)	Vomiting and repetitive falls, pneumonia	6.4×10^9/L	HC-CDI	Bloody	Absent	Amoxicillin and clavulanic acid	No* (symptomatic treatment for diarrhoea)	Diarrhoea resolved
4	$A^-B^-CDT^+$ (NRC, PCR on colonies)	XIb	03/11/2012	04/12/2012	56	Male	Nephrology/ Hôpital Pellegrin	Bordeaux (South of France)	Acute renal failure and pneumonia	13×10^9/L	HC-CDI	PMC	ND	Amoxicillin and clavulanic acid	MTZ po 250 mg 3 times a day	Diarrhoea resolved
5	B^-CDT^+ (Xpert *C. difficile* Assay, Cepheid)	XIa	04/02/2013	05/02/2013	44	Male	Hematology/ Institut Paoli Calmette	Marseille (South of France)	Febrile diarrhoea	25×10^9/L	CO-HC-CDI	Unknown	ND	Ticarcillin and clavulanic acid (+cancer chemotherapy)	MTZ po 500 mg 3 times a day	Death not related to CDI
6	$A^-B^-CDT^+$ (prevalence study)	XIb	10/09/2012	18/09/2012	73	Male	Hepatology-gastroen-terology/ Hôpital Saint Antoine	Paris (North of France)	Worsening of general conditions with hepatocellular carcinoma	5.2×10^9/L	HC-CDI	Unknown	Absent	Unknown	No	Death not related to CDI

ND, not done; HC, health care associated; CDI, *C. difficile* infection; CO, community onset; PMC, pseudomembranous colitis; MTZ, metronidazole; NRC, national reference centre.
**Clostridium difficile* has been isolated only by the NRC during EUropean, multicentre, prospective biannual point prevalence study of *Clostridium difficile* Infection in hospitalized patients with Diarrhoea Euclid study. *C. difficile* testing had not been requested by the physician nor done by the laboratory. Because this study was noninterventional, the result was not immediately transmitted to the physician.

the only *C. difficile* toxinotype to be positive for binary toxin but negative for TcdA and TcdB production ($A^-B^-CDT^+$) (http://www.mf.uni-mb.si/tox/images/Table1_Toxinotypes-characteristics.pdf).

Uncertainties still remain about the clinical significance of toxinotype XI in the pathogenesis of CDI because the role of binary toxin as a virulence factor is still controversial [18]. This toxin is produced by the highly virulent NAP1/BI/027 clone, which has caused severe outbreaks in North America and Europe, and by the emerging 078 clone, suggesting that it may serve as an additional virulence factor and may act in synergy with the large clostridial toxins [3]. A few clinical and epidemiological studies that compared data from infections with strains producing binary toxin in addition to TcdA and TcdB and infections with strains producing only TcdA and TcdB suggested that there could be a correlation between the production of binary toxin and the severity of CDI [23–25]. A case–control study conducted in 2005 compared the clinical presentation of 26 patients infected with strains producing binary toxin in addition to toxins A and B to 42 controls infected with strains producing only toxins A and B. Using univariate analysis, diarrhoea due to a binary toxin–positive strain was more often community acquired ($p = 0.017$) and associated with abdominal pain ($p = 0.07$) than diarrhoea due to binary toxin–negative strains. Diarrhoea was more often the cause of hospitalization in cases than in controls ($p = 0.003$) [23]. A more recent study reported that patients infected with *C. difficile* harbouring genes for toxins A and B and binary toxin had higher case-fatality rates than patients infected with *C. difficile* harbouring genes for toxin A and B without binary toxin [24]. Another study has shown that the presence of the binary toxin gene in *C. difficile* isolates was the only independent predictive factor for recurrent CDI [25]. The effects of binary toxin in animal models gave some conflicting results. In the rabbit ileal loop model, inoculation of supernatants from culture of binary toxin–positive strains that produced neither TcdA nor TcdB leads to enterotoxic response, suggesting that binary toxin may act as a virulence factor. Nevertheless, in the hamster model of ileocolitis, these strains produced no symptoms despite colonization [26]. Recent *in vitro* experiments have shown that binary toxin induces redistribution of microtubules and formation of long microtubule-based protrusions at the surface of intestinal epithelial cells. The CDT-induced microtubule protrusions form a dense mesh work at the cell surface, which wrap and embed bacterial cells, thereby largely increasing the adherence of *C. difficile* [27]. Finally, the importance of binary toxin was demonstrated using isogenic toxin mutants ($A^-B^-CDT^+$) of NAP1/027/BI strains producing only binary toxin inoculated to the hamsters [28]. Three of nine hamsters inoculated with this $A^-B^-CDT^+$ died.

PCR ribotype 033 strains have been reported in animals. In Australia, this PCR ribotype is the second most common strain isolated in cattle and calves [29]. However, very few cases of human CDI due to toxinotype XI have been described in the literature. Among the five cases reported by Geric *et al.* [17], three were from asymptomatic patients and one was from a symptomatic patient; no clinical information was available for the remaining case. In the present report, *C. difficile* strains were isolated from six symptomatic patients with several risk factors for CDI, including advanced age and previous broad-spectrum antibiotic treatment. Four of six patients were successfully treated with metronidazole. These data support the conclusion that diarrhoea in four patients was really due to *C. difficile*.

Only a few studies have estimated the prevalence of toxinotype XI strains. In this study, we found a prevalence of 0.5% (1/220). In a European study, 411 clinical *C. difficile* isolates from 38 hospitals in 14 European countries were characterized by toxinotyping. A total of 354 isolates (86.1%) were toxigenic. Among the toxigenic isolates, 268 (75.7%) were from toxinotype 0; 86 strains (24.3%) belonged to nine variant toxinotypes, but none was of toxinotype XI [15]. A large collection (5000 isolates) of *C. difficile* isolates from the United States and other sources over a 20-year period was examined for the presence of binary toxin genes among strains that do not produce TcdA and TcdB. Eight isolates have been reported as A^-B^- but CDT^+ [17]. Among these eight isolates, five had a truncated PaLoc characteristic of toxinotype XI (including two toxinotype XIa and three toxinotype XIb isolates). However, most of the toxinotype XI strains isolated in that study were recovered from asymptomatic patients. All these data indicate that the prevalence of strains from toxinotype XI in humans seems very low. However, we hypothesize that the prevalence is likely underestimated. Indeed, the microbiological diagnosis of CDI in France is mostly based on the detection of TcdA or TcdB in stool samples, and clinical laboratories do not specifically look for binary toxin. As a consequence, strains from toxinotype XI would be likely reported as nontoxigenic isolates when tested in a clinical laboratory. Since 2009, nucleic acid amplifications assays have been increasingly used for the diagnosis of CDI. Most of the assays only target the *tcdA* or *tcdB* genes of *C. difficile*. Among these tests, the Xpert® *C. difficile* assay simultaneously detects three targets, including *tcdB*, binary toxin genes and the deletion in position 117 within the *tcdC* gene. This assay was initially developed in order to presumptively identify CDI due to the epidemic strain NAP1/BI/027 [3,30]. In recent years, this assay has been widely used in many laboratories in France for the routine diagnosis of CDI. Three cases of toxinotype XI that we report here were detected in clinical laboratories using the Xpert *C. difficile* assay and were suspected only because of the presence of binary toxin gene. It

is likely that the increasing use of this test will enable a better recognition of toxinotype XI and therefore of its potential role in clinical disease.

In conclusion, the present study suggests that *C. difficile* strains producing only binary toxin seem to be pathogenic despite the lack of TcdA and TcdB. A result of *C. difficile* testing that would be only positive by PCR for binary toxin should be taken into account and may reveal a toxinotype XI strain. However, the prevalence of toxinotype XI seems low in France.

Conflict of interest

None declared.

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