ORIGINAL ARTICLE

Clonal dissemination of a toxin-A-negative/toxin-B-positive *Clostridium difficile* strain from patients with antibiotic-associated diarrhea in Poland

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Objective To determine the incidence of toxin-A-negative/toxin-B-positive *Clostridium difficile* strains and their genetic relatedness in the feces of patients suffering from antibiotic-associated diarrhea (AAD) in Polish hospitals.

Methods *C. difficile* strains were cultured from patients' stool samples. The present study characterises these strains with respect to their cytopathogenicity on McCoy cells and the absence of toxin A despite a functional toxin B as determined with commercial test kits (Culturette Brand Toxin CD-TCD toxin A test and *C. difficile* Tox A/B test). In addition, PCR using different primer pairs aiming at non-repeating or repeating regions of the toxin A and B genes were used to confirm the findings. All toxin A^-B^+ strains were genetically characterised by random amplification of polymorphic DNA (RAPD) analysis, PCR ribotyping and, in part, pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments.

Results We here present the presence of 17 toxin A^-B^+ strains among 159 *C. difficile* strains (11%) isolated from fecal samples from 413 patients with antibiotic-associated diarrhea. All 17 strains possessed the toxin B gene, demonstrated a cytopathogenic effect on the McCoy cells, and were positive in the Tox A/B test. Molecular typing of these 17 *C. difficile* strains revealed that 7 of 17 (41%) toxin A^-/B^+ *C. difficile* strains could not be discriminated. It appeared that these strains had a genotype that could not be distinguished from that of a Japanese control strain.

Conclusion Our observations imply that a particular genotype of toxin A^-B^+ *C. difficile* has spread extensively, not only in Poland but possibly even worldwide.

Keywords *Clostridium difficile*, toxin A^-B^+ phenotype, RAPD, PCR ribotyping, PFGE, clonal dissemination

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INTRODUCTION

Clostridium difficile, an anaerobic pathogen encountered in animal and human enteric diseases, generally produces two major virulence factors: exotoxins A and B. These toxins are the largest bacterial toxins known (molecular weights 308 000 and 279 000, respectively). Toxin A is a potent enterotoxin, lethal and cytotoxic, and probably causes most of the clinical symptoms as an immediate consequence of induced fluid secretion and significant mucosal damage. Toxin B is approximately 1000 times more cytotoxic than toxin A [1]. Toxins A and B activate the release of cytokines from human monocytes, and this effect is responsible for colon inflammation in pseudomembranous colitis (PMC) [2–4]. Different PCR assays for the detection of repeating [5] and non-repeating [6,7] domains in the *C. difficile* toxin genes were described for diagnostic purposes. Non-toxigenic strains of *C. difficile* lacking either or both of the genes for toxin A and B are generally considered to be non-pathogenic. However, a number of intriguing questions remain with regard to the pathogenicity of these toxin A^-/B^+ *C. difficile* strains, one of which appeared to be lethal when introduced into an animal model [8]. Moreover, in 1999, an outbreak of toxin A^-B^+ *C. difficile*-associated diarrhea in a Canadian tertiary-care hospital was described [9]. A certain prevalence of toxin A^-B^+ *C. difficile* strains was described in the UK as well [10]. Similar *C. difficile* strains were isolated from fecal samples of patients with antibiotic-associated diarrhea (AAD) in Poland [11]. Toxin A^-/B^+ *C. difficile* strains have deletions in the region

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of the repetitive domain in the toxin A gene, which encodes the epitope that is responsible for the reaction with the antiserum against native toxin A [12]. The aim of the present study was to define the incidence of toxin A^-B^+ *C. difficile* strains among patients suffering from AAD and to analyze the toxicity and genetic heterogeneity of these strains.

MATERIALS AND METHODS

Stool samples

Stool specimens ($n = 413$) were obtained for routine surveillance cultures from as many patients as possible suffering from AAD and hospitalised in different wards of various hospitals in Warsaw between 1995 and 1999. All patients were pretreated with antibiotics and suffered from watery diarrhea and abdominal pain. Among the patients, 60 were nursed on a single transplantation ward, 34 from orthopedics, 62 from internal medicine, 36 from surgery, 12 from intensive care, and 79 from pediatrics, whereas the remaining 130 patients were nursed in various other departments, including gynecology, dermatology, the outpatient clinic and others. Stool samples were tested for the presence of *C. difficile* toxins A and B and were cultured for isolation of *C. difficile*.

Isolation of bacterial strains

Fecal samples were cultured on selective Columbia agar with cycloserine–cefoxitine–amphotericin B (CCCA medium, bioMerieux, Marcy-l'Etoile, France), as described previously [13]. Plates were incubated anaerobically at 37 °C for at least 96 h. Isolates were identified as *C. difficile* by the characteristic morphology of colonies, specific horse odor, green–yellow fluorescence under UV light, Gram staining and biochemical tests (API-20 A, bioMerieux).

Strains

Seventeen suspected toxin A^-B^+ strains of *C. difficile* were isolated from patients with AAD. Three reference strains were included for comparative reasons. The reference set consisted of a single toxigenic strain (VPI 10463), a nontoxigenic isolate (NIH BRRIGS 8050), and one toxin A^-B^+ strain of *C. difficile*, isolated by Dr H. Kato (strain GAI 95601, Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Japan).

EIA detection of *C. difficile* toxins A and B

For detection of toxins A and B, one colony was cultured anaerobically in BHI medium for 4 days. Supernatants were collected by centrifugation (3000 *g* for 15 min) and filtered through 0.45 μm membranes. Toxin A was detected by enzymatic immunoassays. The Culturette Brand Toxin CD-TCD toxin A test (Becton-Dickinson, Meyland, France) was performed according to the manufacturer's instructions. Briefly, horseradish peroxidase-conjugated anti-*C. difficile* toxin A polyclonal antibody and 100 μL of supernatant were added to the microwells and incubated at 37 °C for 1 h. The microwells were washed, and chromogenic substrate was added and incubated at room temperature for 10 min. Reactions were stopped by adding one drop of stop solution and read visually. For detection of either or both of the toxins, Tox A/B *C. difficile* tests (TechLab, Inc., Blacksburg, VA, USA) were performed according to the manufacturer's instructions. One drop of conjugate per microwell was added, followed by the addition of two drops of tested sample prepared as described above. Microwells were incubated at 37 °C for 50 min and washed five times with wash solution. One drop of tetramethylbenzidine substrate was added, followed by one drop of hydrogen peroxide in citric acid buffer. After a 10 min incubation at room temperature, reactions were stopped by adding one drop of stop solution. Test results were interpreted by visual reading: a colorless result was considered negative; any yellow color was considered positive.

Toxin B detection on McCoy cells

In vitro cytotoxin B determination was performed with McCoy cells cultured as described previously [11]. One colony of a *C. difficile* strain was used to inoculate BHI medium and incubated anaerobically. Supernatant fluids were collected as described above. Tenfold serial dilutions of culture filtrate were added in duplicate to McCoy cells and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. The cytopathogenic effect (CPE) was observed by inverse microscopy [14]. If this CPE could be neutralised by polyclonal goat anti-*C. difficile* toxin B antiserum, the test was considered positive.

Genotyping of *C. difficile* strains by arbitrary primed PCR and PCR ribotyping

AP-PCR was performed using two independent arbitrary primers AP-1 and AP-7 in separate assays. PCR-mediated ribotyping employed the consensus primers SP1 and SP2. Both PCR tests were performed as described previously [15].

PCR assay for the detection of the toxin A and B genes

For straightforward detection of the toxin A and toxin B genes, PCR was performed by use of the YT28/YT29 and YT17/YT18 primer sets, respectively, as described previously [7,14]. The cycling conditions for both PCRs were: one predenaturation cycle at 94 °C for 2 min, followed by 94 °C for 45 s, 55 °C for 30 s, and 70 °C for 45 s, for 35 cycles.

PCR assays for the detection of variants of the toxin A gene

PCR amplification aiming at the repeats in the toxin A gene was performed as described by Kato *et al.* [12]. Briefly, 2 μL of DNA solution obtained as described previously [13] was added to 22.5 μL of Supermix (Gibco, BRL, Karlsruhe, Germany), and 1 μL of each primer solution (NK9/NKV011). The cycling conditions applied during PCR were 95 °C for 20 s and 60 °C for 2 min for 40 cycles.

Pulsed-field gel electrophoresis

PFGE was performed by a method described previously [16]. *C. difficile* strains were grown anaerobically on Brucella blood agar at 37 °C for 48 h. Between two and five colonies were embedded in 1% agarose (InCert agarose; FMC BioProducts, Rockland, ME, USA). Each agarose plug was transferred to a sterile cup with 1 mL of proteolytic buffer and incubated overnight at 37 °C. Restriction was performed by placing a 5 mm slice of each plug into 120 μL of restriction buffer for 2 × 30 min and finally in 120 μL of restriction mixture with the appropriate restriction enzyme. Macrorestriction of the DNA was accomplished with *Sma*I or *Nru*I (Boehringer Mannheim, Mannheim, Germany). Restriction fragments were separated by electrophoresis in a 1% Seakem-GTG agarose gel (FMC Bio Products) in 0045 M Tris-HCl, pH 7.5, 0045 M boric acid, 0001 M EDTA. The run time was 20 h at 6 V/cm, with a linearly ramped pulse time (0.5–40 s).

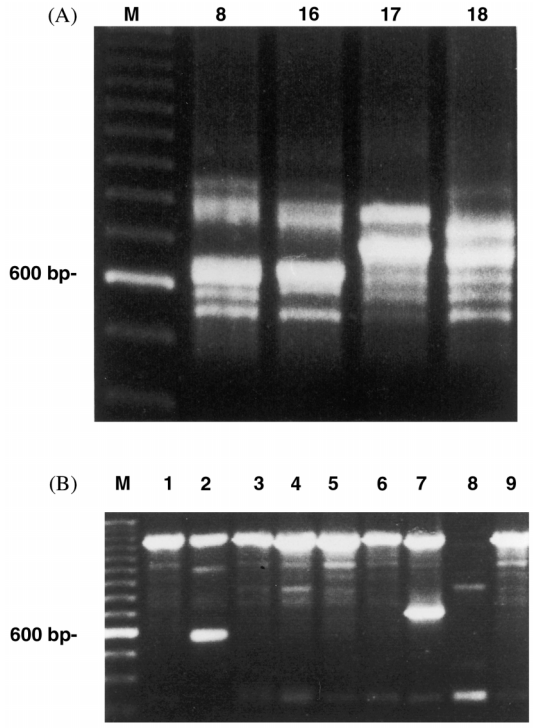

Figure 1 Genotypic characterisation of *C. difficile* strains. (A) Results of PCR ribotyping for four different strains. Strain numbers are indicated above the lanes and the corresponding types are A, H, I and A from left to right. (B) Results of an RAPD test employing primer AP7. Note that the banding patterns for strains 1, 3, 4, 5, 6 and 9, representing the prevalent A-type, are indiscriminate. On the left of both panels, the 600-bp DNA size marker band is indicated (M: 100-bp size ladder (Bio-Rad, Veenendaal, The Netherlands)).

RESULTS

From the 413 stool samples *C. difficile* could be cultured in 159 cases (39%). Of these strains, 120 were toxin A^+ as demonstrated by use of the TCD test. Of the remaining 39 strains, 17 showed a clear CPE on the McCoy cells. For these 17 strains, which were isolated throughout the entire study period from patients nursed in various departments (no-outbreak situation), the results of *C. difficile* toxin testing are presented in Table 1. All strains were cytotoxic for McCoy cells, an effect that could be neutralised by polyclonal antiserum. Interestingly, there was no apparent difference in the severity of the CPE caused by culture supernatants of toxin A^-B^+ or toxin A^+B^+ *C. difficile* strains. This contrasts with earlier reports [8,17]. Toxin A could not be detected by the TCD toxin A test. Toxin A/B tests gave positive results for all 17 clinical cases. The conserved regions of both toxin genes were detectable in all of the Polish isolates studied by the diagnostic PCR tests. The PCR product obtained upon amplification of the non-repeating unit was the same for all toxin A^-B^+ strains and the reference strains.

By PCR ribotyping, we distinguished nine different types among the 17 A^-B^+ *C. difficile* strains. Results of AP-PCR were concordant with those obtained by PCR ribotyping, although a smaller number of types was identified. Altogether, 11 types were distinguishable on the basis of combined PCR ribotyping and AP-PCR (three-letter code in Table 1; for examples of experimental data, see Figure 1). Seven strains, however, were clustered on the basis of the ribotyping and AP-PCR data. These strains were not linked in time or place and were derived from children as well as from adults. These individuals were nursed in a variety of departments, including hematology, gastroenterology, neurology, internal medicine, surgery and orthopedics. Interestingly, the Japanese reference strain showed the same 'A-type'. PFGE was frustrated by the fact that eight strains were untypable, which confirmed similar difficulties for toxin A^+B^+ strains as described by other investigators. Although DNA degradation could not be prevented by formaldehyde treatment of bacteria, it might still be worthwhile for future attempts to prepare the agarose plugs using younger cultures [18]. It is interesting to note, however, that toxin A^-B^+ strains behave similarly to toxin A^+B^+ strains as far as PFGE is concerned. PFGE of the other nine strains identified three and five different types using *Nru*I and *Sma*I enzymes, respectively. Both reference strains, toxigenic and non-toxigenic, were

Table 1 Characterisation of Polish toxin A⁻B⁺ *C. difficile* strains

		TCD[a]	A/B[b]	McCoy[c] Cytotoxicity	Toxin PCR[d] A	Toxin PCR[d] B	RAPD AP1	RAPD AP7	PCR ribotyping	PFGE *Nru*I	PFGE *Sma*I
1.	2428/95	–	+	10^{-5}	+	+	A	A	A	A	A
2.	428/97	–	+	10^{-6}	+	+	A	B	B	B	B
3.	1236/97	–	+	10^{-6}	+	+	A	A	C	NT	NT
4.	1461/97	–	+	10^{-2}	+	+	A	A	D	NT	NT
5.	2785/97	–	+	10^{-3}	+	+	A	A	A	A	C
6.	2887/97	–	+	10^{-3}	+	+	A	A	A	A	A
7.	399/98	–	+	10^{-5}	+	+	A	C	A	A	A
8.	570/98	–	+	10^{-3}	+	+	B	D	E	NT	NT
9.	592/98	–	+	10^{-3}	+	+	A	A	A	A	A
10.	1110/98	–	+	10^{-3}	+	+	A	A	A	NT	NT
11.	2233/98	–	+	10^{-4}	+	+	A	A	A	NT	NT
12.	2560/98	–	+	10^{-3}	+	+	A	E	F	NT	NT
13.	2601/98	–	+	10^{-3}	+	+	A	A	A	A	A
14.	3120/98	–	+	10^{-4}	+	+	A	A	G	NT	NT
15.	205/99	–	+	10^{-1}	+	+	C	G	A	A	D
16.	558/99	–	+	10^{-3}	+	+	B	F	H	C	E
17.	P183/99	–	+	10^{-1}	+	+	A	A	I	NT	NT
18.	GAI 95601[e]	–	+	10^{-3}	+	+	A	A	A	A	A
19.	VPI 10463[f]	+	+	10^{-3}	+	+	A	A	C	D	F
20.	IH BRIGGS 8050[g]	–	–	–	–	–	A	A	J	E	G

[a]TCD, Culturette Brand Toxin CD *C. difficile* toxin A (Becton Dickinson, Franklinokes, NJ, USA); [b]*C. difficile* TOX A/B TEST; [c]last dilution of supernatants, demonstrated cytopathic effect; [d]toxin A and B genes were detected in PCR by using YT-28/YT-29 and YT-17/YT-18 primers, respectively; [e]*C. difficile* toxin-A-negative toxin-B-positive-strain isolated in Japan; [f]toxigenic reference strain; [g]non-toxigenic reference strains; NT, non-typable.

confirmed to be completely different types. Four strains (numbers 1, 6, 9 and 13) were clonally related, belonging to the already mentioned A-type. One strain (number 5) demonstrated single-fragment differences with the A-type when *Sma*I was used. The Japanese *C. difficile* strain belonged to the A-type. For nine toxin A⁻B⁺ *C. difficile* strains, PCR amplification with the NK9/NKV011 primer set generated a PCR product (700 bp) similar to that obtained for the Japanese GAI 95601 strain. Interestingly, all clinical strains that displayed ribotype A had a deletion in the A gene. PCR analysis of the remaining eight toxin A⁻B⁺ *C. difficile* strains revealed an amplicon with the expected size of approximately 2500 bp.

DISCUSSION

We analysed fecal samples of a large group of patients suspected of having AAD over a 5-year surveillance period. In 39% of all samples, *C. difficile* was encountered and, to our surprise, 11% of these isolates failed to express toxin A. These 17 toxin A⁻B⁺ *C. difficile* strains were negative in the TCD immunoassay and positive in the Tox A/B test, designed for detection of both toxins. All 17 strains demonstrated a cytopathogenic effect on McCoy cells, and this effect was neutralised by *C. difficile* antitoxin. Both toxin genes were detected in our 17 strains, the toxigenic reference strain and the Japanese toxin A⁻B⁺ strain. Only the non-toxigenic reference strain lacked both toxin genes. It is well known that both toxins of *C. difficile* possess repeating units at the C-terminus. The repeating units of the toxin A represent the part recognised by the monoclonal antibody. Deletions in the repeating sequences of the toxin gene in toxin A⁻B⁺ strains were demonstrated by Kato and coworkers [12]. Similar deletions as identified by PCR were the reason why our strains demonstrated negative reactions in the toxin A TCD enzymatic test. Toxin A⁻B⁺ strains without the overt deletion in the repeat region were shown to produce minute amounts of toxin A: more than twofold concentration of culture supernatants produced a small signal (results not shown). It could be that this represents read-through products of a mutated toxin A gene, as described by Von Eichel-Streiber *et al.* [19]. We have not yet confirmed this by sequence analysis (work in progress). Recently, Japanese authors described a PCR with specific primers for detection of toxin A⁻B⁺ *C. difficile* strains directly in stool samples, a valuable tool for future epidemiologic studies [17].

In this study, we investigated unrelated, clinical *C. difficile* isolates from different hospital units found at different moments in time. Nevertheless, we found seven toxin A⁻B⁺ *C. difficile* strains belonging to a single genogroup A, being similar to the Japanese strain (number 18, Table 1). The clonal isolates, furthermore, share high-level resistance against clindamycin

and harbor the *erm*B gene (results not shown). This strongly suggests widespread dissemination of certain clones of toxin A^-B^+ *C. difficile*, which emphasises that additional studies on the role and mechanisms of intercontinental spread of these apparently pathogenic bacterial pathogens should be undertaken.

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