Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive Clostridium difficile strains

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Abstract

The repeating sequences of the toxin A gene from toxin A-negative, toxin B-positive (toxin A−, toxin B+) strains of Clostridium difficile which were isolated in geographically separated facilities in Japan and Indonesia were determined. All six strains tested had identical repeating sequences with two deletions (1548 and 273 nucleotides in size) in the toxin A gene. A PCR method was designed to detect the deletions and the deletions were confirmed in all 50 toxin A−, toxin B+ strains examined by this method. Western immunoblot analysis revealed that polyclonal antiserum against native toxin A did not react with the concentrated culture filtrates of the toxin A−, toxin B+ strains. These results may suggest that toxin A−, toxin B+ strains have deletions of the two thirds of the repeating regions of the toxin A gene, which encodes the epitopes fully responsible for the reaction with the polyclonal antiserum.

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1. Introduction

Clostridium difficile, a principal pathogen causing antibiotic-associated diarrhea and colitis, is recognized as an important nosocomial pathogen. Toxins A (potent enterotoxin) and B (potent cytotoxin) produced by this organism play a major role in its enteropathogenicity [1,2]. The two toxins show 63% amino acid sequence homology and carry repetitive C-termini [3]. Nearly a third of the toxin A gene, at the 3’ end, consists of 38 repeating nucleotide units [4] and the repetitive amino acid sequences encoded by repeating nucleotide units contain the cell-binding domain [5,6] and the enzymatic domain of toxin A is located in its N-terminal region [7]. The C-terminal amino acid regions also have epitopes recognized by the monoclonal antibody (mAb) PCG-4 [8] which is widely used in commercial en-
zyme-linked immunosorbent assay (ELISA) kits for toxin A detection [9].

All toxigenic strains were believed to produce both toxins A and B, but this has been found not always to be true [10–13]. We successfully used a PCR assay (with a primer set NK11-NK9) targeted to the repeating sequences of the toxin A gene to distinguish the strains which are toxin A-negative, toxin B-positive (toxin A−, toxin B+) strains from toxin A-positive, toxin B-positive (toxin A+, toxin B+) strains and toxin A-negative, toxin B-negative strains (toxin A−, toxin B−) [13]. By the PCR with NK11-NK9 primers, whereas toxin A+, toxin B+ strains generated an approximately 1200-bp product, a shorter segment of ca. 700 bp was amplified from toxin A−, toxin B+ strains [13], suggesting a constitutive difference of the repetitive region between toxin A−, toxin B+ strains and toxin A+, toxin B+ strains [13].

In this study, we determined the whole repeating sequences of the toxin A gene of six toxin A−, toxin B+ strains. In addition, reverse transcriptase (RT)-PCR was carried out to examine the transcription of the nonrepeating regions of the toxin A gene in toxin A−, toxin B+ strains and immunoblotting analysis was performed to detect a toxin A variant, if produced, with a polyclonal antiserum against native toxin A.

2. Materials and methods

2.1. Bacterial strains

C. difficile strains used for sequencing analysis were the reference strain of serogroup F, ATCC 43598, and five toxin A−, toxin B+ clinical isolates which were isolated in geographically separated facilities in Japan and Indonesia: GAI 95601 and GAI 95603 from asymptomatic adults, GAI 95602 from a symptomatic adult, GAI 95600 and GAI 95606 from asymptomatic children. All strains were positive for toxin B by cell culture assay and negative for both toxin A by ELISA using monoclonal antibody and an ileal loop test in rabbits as reported previously [13]. All strains except for one (GAI 95606, serogroup X) were of serogroup F [14]. A total of 50 toxin A−, toxin B+ C. difficile strains isolated in various clinical settings were examined by PCR assay to confirm the deletion of the repeating regions of the toxin A gene. G95-13 was used as a representative of toxin A−, toxin B− strains.

2.2. Sequencing analysis

Bacterial genomic DNA was extracted and purified as described elsewhere [15]. Primers used for sequencing analysis are listed in Table 1. Primers NK16 and HK42 were originated at 330 nucleotides upstream from the beginning of the repeating sequences and at 26 nucleotides downstream from the stop codon, respectively, based on the published sequence of the toxin A gene in VPI10463 [4]. Primers NKV45 and NKV 46 were obtained from the results of sequencing for GAI95601.

PCR amplification was performed as described previously [16,17]. The thermal profile was 35 cycles comprising 95°C for 20 s for denature and 55°C for 2 min for annealing and extension. After electrophoresis, PCR products were purified by using PrepA-Gene purification matrix kit (Bio-Rad, Richmond, CA, USA) and sequenced directly with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and 373 automatic DNA sequencer (PE Applied Biosystems). The DNA sequence analysis was performed by DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

2.3. Nucleotide sequence accession number

The repeating sequence of strain GAI95601 has been registered at the DDBJ database (accession number AB012304).

2.4. PCR assay to detect the deletion of the repeating regions of the toxin A gene

PCR amplification was performed as described [13,17] for 35 cycles with modifications. Briefly, 1 µl of DNA solution obtained by heat treatment of a bacterial colony was added to 30 µl of a reaction solution comprising 0.75 U of TaKaRa Ex Taq (TaKaRa, Shiga, Japan), Ex Taq reaction buffer (TaKaRa), dNTP mixture (200 µM each), 4.5 ng of each primer. Primers used for PCR are shown in
Table 1. A hot start PCR using AmpliWax PCR Gem (TaKaRa) was carried out to obtain primer-specific amplification. The cycling conditions for primer pair NK11-NK9 were 95°C for 20 s followed by 62°C for 2 min. PCR with primer pair NKV011-NK9 was performed in a condition consisting of 95°C for 20 s and 60°C for 2 min.

2.5. RT-PCR analysis of transcription of the nonrepeating regions of the toxin A gene

*C. difficile* VPI10463 and GAI95601 were cultured in each 10 ml of brain heart infusion (Difco Laboratories, Detroit, MI, USA), harvested at the early stationary phases of growth. Bacterial cells were suspended in 5% lysozyme (Sigma, St. Louis, MO, USA) in TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and frozen at −75°C for more than 1 day. Following thawing at 37°C, bacterial total RNA was extracted by using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. After ethanol precipitation following phenol/water/chloroform extraction, RNA samples were treated with 5 U µl⁻¹ of DNase (Sigma) to remove contaminating DNA. Samples were again treated with phenol/water/chloroform followed by ethanol precipitation. Reverse transcription using 2 µg of total RNA was performed with SuperScript Preamplification System (Gibco BRL Life Technologies, Rockville, MD, USA) with random hexamers as per the manufacturer’s instructions. PCR amplification was carried out with primers NK12 and NK2 (117-bp product) for a fragment of the nonrepeating region of the toxin A gene [17]. PCR with primers NK104 and NK105 was run with the same RNA sample to amplify a fragment of 204 bp of the toxin B gene [13], as transcription control. PCR amplification was carried out with or without RT during the initial cDNA generation to prove the non-DNA contamination in RNA samples tested. PCR products were analyzed with a 5% polyacrylamide gel electrophoresis (PAGE) followed by ethidium bromide staining.

2.6. PAGE and immunoblotting

Strains of VPI10463, GAI95601, GAI95600, GAI95606, and G95-13 were grown anaerobically at 37°C for 72 h with the brain heart infusion dialysis flask technique [18]. Precipitant of supernatant with ammonium sulfate was harvested by centrifugation, redissolved in 6 M urea in TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl), and dialyzed against TBS. The immunoblotting was performed as described previously [14]. The concentrated culture supernatant (15 µg of total protein) and purified native toxin A were separated by 5% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), and probed with a 1:500 diluted goat polyclonal antiserum against toxin A.

**Table 1**

<table>
<thead>
<tr>
<th>Primer used in this study</th>
<th>Orientation</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer for sequencing</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NK16 forward</td>
<td></td>
<td>TGTTCTACAGAGGAAGTGGA</td>
<td>5215-5234^a</td>
</tr>
<tr>
<td>HK42 reverse</td>
<td></td>
<td>TAAATTCTTATAGCTACAGGG</td>
<td>8159-8178^b</td>
</tr>
<tr>
<td>HKV45 forward</td>
<td></td>
<td>TTTGAGTATTGTGACCTGGC</td>
<td>160-179^b</td>
</tr>
<tr>
<td>HKV46 reverse</td>
<td></td>
<td>CATCCAGTAGCTGCTCAAGC</td>
<td>337-356^b</td>
</tr>
<tr>
<td>Primer for PCR assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK11 forward</td>
<td></td>
<td>TGATGCTAAATGGAAATCTAAAATGTTAAC</td>
<td>6795-6824^c</td>
</tr>
<tr>
<td>NK9 reverse</td>
<td></td>
<td>CCACGCCGCTGACCCATA</td>
<td>8043-8060^b, 678-695^b</td>
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<td>NKV011 forward</td>
<td></td>
<td>TTTTGATCTTATAGCTAACTTAGTAAC</td>
<td>−19-11^b, 5526-5555^c</td>
</tr>
</tbody>
</table>

^aThe toxin A gene in VPI10463 [4].
^bThe repeating sequence of the toxin A gene in GAI95601 (this study).
^cNKV011 primer has a mismatch of one nucleotide with the toxin A gene of VPI10463, 5’-TTTTGATCTATAGGATCTAACTTAGTAAC-3’, as indicated with an underline.
3. Results

3.1. Sequences of the repeating portion of the toxin A gene

Nucleotide sequences of the repeating portion of the toxin A gene in GAI95601 was determined. The size of the repeating sequences of GAI95601 was 741 nucleotides. Two in-frame deletion sites, 1548 nucleotides (nucleotide positions corresponding to 5839–7386 in VPI10463) and 273 nucleotides (7690–7962 in VPI10463) in length were found as compared with the corresponding sequence of VPI10463 [4] (Fig. 1). Three homologous regions (shaded boxes, Fig. 1) exhibited high identities (95–99%) between the two strains. Furthermore, sequencing analysis showed that the repeating sequences from four other clinical toxin A− and toxin B+ strains and ATCC 43598 were identical with those of GAI95601.

3.2. Comparison of deduced amino acid sequences

Comparisons of deduced amino acid sequences of the repeating units in GAI95601 and VPI10463 are shown in Fig. 2. In GAI95601, there were 11 repeating units which consisted of two class I units (I1 and I6) and nine class II units according to the classification by Dove et al. [4]. The class II repeating units were composed of two class IIA, four class IIB, two class IIC, and one class IID.

3.3. PCR assay to detect the deletion of the repeating regions of the toxin A gene

Although PCR with NK11-NK9 primers amplified DNA of approximately 700 nucleotides in 50 toxin A−, toxin B+ strains tested, the nucleotide sequences of primer NK11 were not found in the repeating region because of the deletion (Fig. 1). However, a sequence similar to that of primer NK11 was found to exist spanning from the end of the nonrepeating to the beginning of the repeating sequences in GAI95601. Then, primer NKV011 was designed according to the sequence results obtained (Fig. 1). PCR for DNA samples from GAI95601, GAI95600, and GAI95606 generated an amplicon with the same size (approximately 700 bp) using NKV011-NK9 primers as that seen in PCR using NK11-NK9 primers (Fig. 3). Hot start PCR to pro-

Fig. 1. Comparison of the repeating sequences of the toxin A gene in toxin A−, toxin B+ GAI95601 (data from the present study) and those of toxin A+, toxin B+ VPI10463 reported in the previous study [4].
vide more stringent annealing condition yielded an amplicon in GAI95601 with the NKV011-NK9 primer set but not with the NK11-NK9 primer set (data not shown). PCR amplification with the NKV011-NK9 primer set generated a PCR product of the same size as that of GAI95601 for the 50 toxin A\(^3\), toxin B+ strains. Although primer NKV011 has a mismatch of one nucleotide with the toxin A gene of VPI10463 (Table 1), PCR with NKV011-NK9 primers and TaKaRa Ex Taq generated an amplicon with an expected size of approximately 2500 bp in VPI10463 (Fig. 3).

3.4. RT-PCR analysis of the gene transcription

We used RT-PCR to investigate transcription of the nonrepeating regions of the toxin A gene. Examination by PCR of the RNA samples for which reverse transcription was performed revealed that both VPI10463 and GAI95601 transcribed a 117-bp portion of the nonrepeating regions of the toxin A gene.
(data not shown) as well as a segment of 204 bp of the toxin B gene (data not shown). No transcript was observed for the RNA samples of these two strains for which reverse transcription was not performed.

3.5. Western immunoblot analysis

Western immunoblot analysis revealed that the polyclonal antiserum against toxin A does not react with the concentrated culture filtrates from toxin A−, toxin B+ strains while native toxin A and VPI10463 demonstrated the positive band of 308 kDa (Fig. 4).

4. Discussion

The present study revealed two deletions in the repeating sequences of the toxin A gene for six toxin A−, toxin B+ C. difficile strains isolated in different facilities. PCR with a NKV011-NK9 primer set, which specifically amplifies a DNA fragment of almost the entire repeating region of the toxin A gene, revealed a PCR product with an expected size in all of the 50 toxin A−, toxin B+ strains tested. These results suggest that toxin A−, toxin B+ C. difficile strains commonly have identical deletions in the repeating portion and constitute a distinct toxin-producing type. Thus, PCR with the NKV011-NK9 or NK11-NK9 primer set can differentiate toxin A−, toxin B+ strains of C. difficile strains from toxin A+, toxin B+ strains.

Frey et al. reported that mAb PCG-4 recognizes two epitopes, residues 2097–2141 and 2355–2398 in an amino acid sequence [8]. The nucleotide sequences encoding both epitopes were included in the portions that were found to be deleted in toxin A−, toxin B+ strains. These results are consistent with the fact that toxin A−, toxin B+ strains were negative for toxin A by ELISA using mAb PCG-4 [13]. In addition, toxin A−, toxin B+ strains gave negative results in the Western blotting with anti-toxin A polyclonal antiserum, suggesting that the epitopes recognized by the polyclonal antiserum are also located within the C-terminal region of toxin A. In addition, RT-PCR study showed at least in part the transcription of the nonrepeating sequence of the toxin A gene in a toxin A−, toxin B+ strain. But in our study no direct evidence was obtained to prove that the truncated toxin is actually produced from the toxin A of toxin A−, toxin B+ strains since we did not carry out further studies using Northern blot analysis allowing quantitative deletion of the full-length mRNA or Western blotting using antisera against synthesized polypeptides deduced from the sequence of the mutated toxin A gene. Further study using a transcriptional and translational fusion systems would clarify whether toxin A variant is actually produced by toxin A−, toxin B+ strains.

We isolated a toxin A−, toxin B+ strain of C. difficile from a 14-year-old child who suffered from PMC (unpublished data), indicating possible enteropathogenicity of toxin A−, toxin B+ strains in humans. Riegler et al. have demonstrated that the human colon is more sensitive to the damaging effects of toxin B than to the effects of toxin A [19], suggesting that toxin B alone may cause intestinal disorders in the human. Further studies are needed to clarify the clinical significance of toxin A−, toxin B+ strains of C. difficile in humans.

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References

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