Characterisation of *Clostridium difficile* strains by polymerase chain reaction with toxin A- and B-specific primers

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Summary. A total of 218 *Clostridium difficile* strains was examined for production of toxin A by ELISA, production of toxin B by a cytotoxin assay and the presence of toxin A and B gene-associated sequences by the polymerase chain reaction (PCR). After saturation amplification with toxin B-specific primers, the characteristic amplification product (591 bp) was detected in all 184 toxigenic strains examined. PCR with toxin A-specific primers gave positive results with all but one of the toxigenic strains. By contrast, PCR with toxin A- and toxin B-specific primers yielded negative results with all 34 non-toxigenic strains tested. This suggests that PCR detection of either the toxin A or B gene is a good indication of toxin production. PCR did not require DNA extraction or hybridisation and was convenient, sensitive and rapid. Toxigenic *C. difficile* could be detected in mixed cultures, suggesting a role for PCR in the identification of toxigenic *C. difficile* in primary culture.

Introduction

Toxigenic strains of *Clostridium difficile* are the major cause of pseudomembranous colitis and antibiotic-associated colitis and diarrhoea.¹ Two toxins appear to be involved in disease: toxin A, an enterotoxin with cytotoxic activity; and toxin B, a potent cytotoxin.² The diagnosis of *C. difficile*-associated disease depends on the isolation and identification of the organism or the detection and neutralisation of cytotoxic activity from faecal specimens, or both. Results correlate well with clinical disease, but the cytotoxin assay is costly, the toxin cross-reacts with *C. sordellii* toxin, and some laboratories may lack the required facilities. Recently, a direct faecal ELISA (Meridian Diagnostics, Cincinnati, USA), which also cross-reacts with *C. sordellii* toxin, has been reported to have 84.1% sensitivity compared with cytotoxin assay.³ This is a rapid, sensitive but expensive test. Another less costly assay for the presence of toxin A is a latex test (Culturette Brand *C. difficile* test; Marion Laboratories, Kansas, USA) which has been shown to be non-specific for *C. difficile* and to give a large number of false positive results.^{4,5} More rapid, specific and cost-effective tests for the detection of toxigenic *C. difficile* are needed.

The polymerase chain reaction (PCR) allows sensitive detection of a given DNA sequence even in a complex mixture of molecules and has been used in the

identification of a range of pathogenic organisms.⁶ The decrease in cost of thermostable DNA polymerases may make PCR a cost-effective alternative to traditional diagnostic procedures. A potential drawback of PCR in diagnostic applications is that it detects genotypic rather than phenotypic characteristics, and "silent" genes may give false positive results.

Recently, we described a rapid and sensitive assay for toxigenic *C. difficile* by PCR with boiled extracts.^{7,8} The toxin A-specific oligonucleotide primers used were tandem repeat nucleotide sequences from the toxin A gene, which resulted in a characteristic profile of amplified products that provided an easily interpretable alternative to conventional single-product PCR.⁷⁻⁹ Previous reports on PCR-based identification

Fig. 1. Identification of toxigenic *C. difficile* with toxin A-specific primers. Lanes B, D, E, W, X and Z, toxigenic *C. difficile* strains; A, C and Y, non-toxigenic *C. difficile* strains; 1, DNA size markers (*Bsh* 1 and *Msp* 1 digests of pH314).

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of *C. difficile* have used other toxin A- and B-specific primers or 16S rRNA-specific primers,¹⁰⁻¹² but examined only a limited number of strains. The aim of this study was to determine whether the detection of toxin A and toxin B gene fragments by PCR is a reliable indicator of toxin production in a large number of *C. difficile* strains from nine distinct typing groups.¹³ Detection of toxigenic *C. difficile* by PCR was also evaluated in mixed culture with a view to developing an alternative method for the detection of toxigenic *C. difficile*.

Materials and methods

Bacterial strains

C. difficile strains (218) were cultured anaerobically at 37°C on Cycloserine-Cefoxitin-Fructose Agar (Oxoid) and identified by Gram's stain, smell, colony morphology and their characteristic pattern of volatile fatty acid production.¹⁴ Strains were typed by [³⁵S]-methionine-labelled SDS-PAGE protein profiles (table I).¹³ Twenty strains representing 18 other *Clostridium* spp. (table II) were cultured on Columbia Horse Blood agar (Oxoid) and identified by standard procedures.¹⁴ Two streptococcal strains were identified by the API Strep system (API system, Montalieu Vercieu, France).

Toxin assays

C. difficile strains were cultured in Robertson's Cooked Meat Medium (Southern Group Laboratories, London) at 37°C for 3 days and stored at room temperature for subsequent toxin assay. Toxin A was measured quantitatively by ELISA.¹⁵ Toxin B was detected by a cytotoxin assay, after pre-incubation with excess antibody to toxin A.¹⁶

Preparation of PCR samples

Two or three colonies were suspended in 200 µl of sterile water, boiled for 10 min and centrifuged at 14000 g for 5 min; 2 µl of supernate was used for PCR analysis of bacterial samples.

PCR assay

The toxin A primers (5'-GAAGCAGCTACTG-GATGGC and 5'-AGCAGTGTTAGTATTAAG) and toxin B primers (5'-AAGGTTTATATGGAT-GAT and 5'-TACAACCTTTATTAACACAAC), were synthesised on an Applied Biosystems synthesiser by the automated phosphoramidite coupling procedure. For PCR reactions, 2 µl of the supernate from the boiled sample was added to 50 µl of 10 mM Tris-HCl (pH 8.3) containing 1.5 mM MgCl₂, gelatin 0.01 %, 200 µM each of dATP, dCTP, dGTP and dTTP, 100 pM oligonucleotide primers and 1.5 U *Taq* polymerase

(Amersham International). The reaction mixture was overlaid with paraffin oil (50 µl), placed in a thermal cycler (Model 2; Hybaid Ltd, Twickenham, Middlesex) and amplified for 30 cycles of 94°C (45 s), 46°C (45 s) and 72°C (15 s) for toxin A and for 40 cycles of 94°C (45 s), 40°C (45 s) and 72°C (45 s) for toxin B. The first 94°C step was extended to 3 min to ensure denaturation of the sample. After amplification, the products (25 µl) were electrophoresed in a horizontal agarose 1.5% gel containing ethidium bromide 0.5 µg/ml and the bands were visualised under ultraviolet light. All PCR analyses were performed in duplicate with positive (*C. difficile* strain W1) and negative (no organism) controls.

PCR assay of mixed cultures and assessment of assay sensitivity

A single *C. difficile* W1 or *C. sporogenes* NCTC 532 colony was picked, serially diluted in Wilkins Chalgren Broth (Oxoid) and spread on the surface of blood agar plates for incubation at 37°C under anaerobic conditions for 36 h to determine the viable count. Known quantities of toxigenic *C. difficile* and *C. sporogenes* were mixed and used as samples in PCR experiments.

Results

PCR with toxin A primers

Fig. 1 shows PCR results obtained with the tandem repeat sequence toxin A-specific primers. Amplification products appeared as a ladder of bands at multiples of the 63-bp unit length for the toxin-producing strains of SDS-PAGE groups B, D, E, W, X and Z. In contrast, no amplification products were observed from the non-toxigenic strains of SDS-PAGE groups A, C and Y. Of the 184 toxigenic strains examined, only one was PCR-negative (table I), and this anomalous strain (strain 8864) also gave negative results in the ELISA for toxin A (table I).

PCR with toxin B primers

Fig. 2 shows PCR results obtained with the toxin B-specific primers based on a non-repeating region of the toxin B gene.¹⁷ A single amplified product of expected size (591 bp) was observed with toxin-producing strains of SDS-PAGE groups B, D, E, W, X and Z. In contrast, no amplification products were found from the non-toxigenic strains of SDS-PAGE groups A, C and Y. All 184 cytotoxigenic strains yielded the characteristic 591-bp product, but strain 8864 also yielded a product of c. 690 bp.

Other bacterial species

Eighteen other clostridial species were tested for toxin A and B gene sequences by PCR. Toxin B-

Table I. Results of tissue culture, ELISA and PCR-based toxin analyses of typed *C. difficile* strains

Type	Number of strains examined	ELISA result (toxin A)		Tissue culture result (toxin B)		PCR result			
		+	-	+	-	toxin A		toxin B	
						+	-	+	-
A	6	0	6	0	6	0	6	0	6
B	*17	16	1	17	0	16	1	17	0
C	5	2	3	2	3	2	3	2	3
D	7	4	3	4	3	4	3	4	3
E	23	23	0	23	0	23	0	23	0
W	2	2	0	2	0	2	0	2	0
X	140	135	5	135	5	135	5	135	5
Y	17	0	17	0	17	0	17	0	17
Z	1	1	0	1	0	1	0	1	0
Total	218	183	35	184	34	183	35	184	34

*Includes one anomalous toxin A negative, toxin B positive strain.

Fig. 2. Identification of toxigenic *C. difficile* with toxin B-specific primers. Lanes B, D, E, W, X and Z, toxigenic *C. difficile* strains; A, C and Y, non-toxigenic *C. difficile* strains; M, DNA size markers (100-bp ladder).

specific primers failed to amplify DNA from all non-*difficile* species tested, but PCR with toxin A-specific primers yielded amplification products from two toxigenic strains of *C. sordellii* (table II). Two strains representing *S. mutans* and *S. downei*, that are known to produce proteins similar to toxins A and B,¹⁸ gave negative results in PCR with both primer sets (table II).

Sensitivity of PCR

Sensitivity was determined by assay of serial dilutions of a suspension of *C. difficile* W1. Characteristic amplification products remained detectable with as few as 20 cfu of toxigenic bacteria (toxin A) or 50 cfu of toxigenic bacteria (toxin B)/PCR sample (table III). This level of sensitivity was maintained in a

Table II. Results of PCR-based analyses of non-*difficile* clostridium spp. and streptococci

Strain*	PCR result	
	Toxin A	Toxin B
<i>C. beijerinckii</i> NCTC 11920	-	-
<i>C. butyricum</i> NCTC 7423	-	-
<i>C. carnis</i> NCTC 10913	-	-
<i>C. chauvoei</i> NCTC 8070	-	-
<i>C. fallax</i> R2720	-	-
<i>C. histolyticum</i> NCTC 1503	-	-
<i>C. innocuum</i> R657	-	-
<i>C. novyi</i> type A NCTC 538	-	-
<i>C. paraputrificum</i> NCTC 11823	-	-
<i>C. perfringens</i> NCTC 1265	-	-
<i>C. putrefaciens</i> NCTC 9836	-	-
<i>C. septicum</i> NCTC 547	-	-
<i>C. sordellii</i> NCTC 6800	+	-
<i>C. sordellii</i> NCTC 8780	+	-
<i>C. sphenoides</i> NCTC 507	-	-
<i>C. sporogenes</i> NCTC 532	-	-
<i>C. tertium</i> NCTC 541	-	-
<i>C. tetani</i> NCTC 540	-	-
<i>C. tetanomorphum</i> NCTC 548	-	-
<i>S. downei</i> MFe28	-	-
<i>S. mutans</i> GS-5	-	-

*NCTC strains were obtained from the National Collection of Type Cultures, Public Health Laboratory Service, Colindale. *C. fallax* R2720 and *C. innocuum* R657 were obtained from M. Phillips, Public Health Laboratory, Luton. The streptococcal strains were provided by R. R. B. Russell, Department of Oral Microbiology, University of Newcastle upon Tyne.

mixed culture containing 10⁷ cfu of *C. sporogenes* NCTC 532/PCR sample (table III).

Discussion

This study demonstrated that PCR with primers based on either toxin gene sequence can be used as a rapid method to confirm the toxigenic potential of *C. difficile* strains. No false positive PCR results were obtained with 34 non-toxigenic *C. difficile* strains,

Table III. Sensitivity of PCR with toxin A- and B-specific primers for the detection of toxigenic *C. difficile*

Viable counts of <i>C. difficile</i> (cfu/sample)	PCR results with			
	Toxin A primers	Toxin B primers	Toxin A primers with <i>C. difficile</i> in the presence of 10 ⁷ cfu of <i>C. sporogenes</i> / sample	Toxin B primers with <i>C. difficile</i> in the presence of 10 ⁷ cfu of <i>C. sporogenes</i> / sample
5	—	—	—	—
10	+/-*	—	—	—
20	+	—	+	—
50	+	+	+	+
100	+	+	+	+

*Some bands partially visible.

suggesting that "silent" toxin genes are not present among these strains. Among the 218 toxigenic strains tested by ELISA and tissue culture analysis, 217 were PCR positive with toxin A-specific primers and all gave positive results with the toxin B-specific primers. The anomalous strain (strain 8864) was cytotoxic, but did not produce toxin A; this strain has been described elsewhere.¹⁹ Recently, McMillen *et al.*¹⁰ described PCR analysis of 47 *C. difficile* strains with a different set of toxin A- and B-specific primers. From their survey, a single anomalous strain was PCR positive with both sets of primers, but was not cytotoxic under the conditions tested.¹⁰ These studies demonstrate the usefulness of PCR in identifying toxigenic *C. difficile* and mutations at the two toxin loci.

This study confirms that most *C. difficile* strains appear to produce either both toxins or neither toxin.¹⁶ The toxin B gene is located only 1.4 kb upstream of the toxin A gene²⁰ and co-regulation of the two toxins has been observed in a large number of *C. difficile* strains,¹⁶ suggesting that the toxin genes form a transcription unit. Previously we have shown that DNA from non-toxigenic *C. difficile* strains fails to hybridise with a 4.5-kb toxin A gene-specific probe, suggesting that these strains lack at least 4.5 kb of the toxin A gene.²¹ The absence of PCR products from non-toxigenic strains reported here suggests that these strains have a large deletion extending across both toxin genes. It was noticeable that non-toxigenic strains were predominantly SDS-PAGE typing groups A or Y, and these may represent a distinct sub-species of *C. difficile*.

The use of the tandem repeat sequence toxin A-specific primers in PCR has two advantages over the toxin B-primers. First, a characteristic profile of bands is amplified from the tandem repeat primers providing a more easily interpretable result. Secondly, the toxin A-specific primers give a PCR assay at least two and a half times more sensitive than the toxin B-specific primers. In contrast to the non-repeat toxin B primers, toxin A primers amplified DNA from toxigenic strains of *C. sordellii*. However, this cross-reaction probably results from shared genetic sequences, rather than being intrinsic to the tandem sequence primer design.

The PCR assay for the identification of toxigenic *C. difficile* described here is rapid, sensitive and requires minimum specimen preparation. Results could be obtained within 3 h of primary isolation and PCR was successful even in mixed culture with *C. sporogenes*. Gumerlock *et al.* have described a method for the isolation of *C. difficile* DNA from human stool specimens and were able to detect *C. difficile* from these samples with 16S rRNA-specific primers.¹² However, they were unable to distinguish between toxigenic and non-toxigenic strains. The use of the toxin-specific primers described in this study coupled with the preparation method described by Gumerlock *et al.*¹² could provide a cost-effective PCR-based method for the direct detection of toxigenic *C. difficile* from human faeces.

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