Detection of *Clostridium difficile* enterotoxin gene in clinical specimens by the polymerase chain reaction

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Summary. A rapid assay was developed for detection of the *Clostridium difficile* enterotoxin gene in stool specimens by means of the polymerase chain reaction (PCR). The PCR primers amplified a 63-bp repetitive sequence of the enterotoxin gene, thereby generating a distinctive ladder pattern of DNA bands following electrophoresis. Crude DNA extracts from stools containing *C. difficile* produced one (63-bp) or more bands of the characteristic ladder. Of 172 stool specimens from 58 patients, 37 gave positive results by culture (15 specimens) or cytotoxin assay (36 specimens). When 36 available "positive" specimens were tested by the PCR assay, 34 (94%) gave positive results—24 by direct testing, and 10 after extraction of DNA by the Qiagen procedure. Insufficient material of the remaining two specimens was available for DNA extraction. Of 21 stools "negative" for *C. difficile* by culture or cytotoxin assay, one gave a positive result by PCR and seven produced atypical bands. The rapid PCR detection technique for *C. difficile* was more sensitive than standard culture, and of a sensitivity similar to cytotoxin testing. The method has the potential for adoption in routine laboratory practice.

Introduction

Pseudomembranous colitis and antibiotic-associated diarrhoea in man are associated closely with *Clostridium difficile*.^{1,2} Two major toxins are produced by *C. difficile*, an enterotoxin and a cytotoxin,^{3,4} of which the enterotoxin is thought to be the main cause of the disease symptoms.⁵ Current culture-based detection methods for *C. difficile* in human stools are slower and less sensitive than detection of cytotoxin; however, the latter requires cell-culture facilities, and is not entirely specific for *C. difficile*. Various ELISA techniques have been developed, but these lack sensitivity, specificity or both.⁶

The *C. difficile* enterotoxin gene has been sequenced, and repeat sequences have been characterised.^{7,8} One of the repeat sequences has been used in conjunction with the polymerase chain reaction (PCR) to demonstrate the enterotoxin gene in isolates of *C. difficile*,⁷ thereby providing the basis for an alternative, highly specific, rapid assay. This paper describes the application of such an assay to the direct detection of the enterotoxin gene of *C. difficile* in clinical specimens.

Materials and methods

Clinical specimens and bacteria

Specimens used in this study were loose or liquid stools from patients at Heidelberg Repatriation Hospital; most of these patients had received antibiotic therapy and were suspected of having *C. difficile*-related diarrhoea. All stool samples were cultured for enteric pathogens, including *C. difficile*, and were tested for *C. difficile* cytotoxin. Stool samples were stored at -20°C before assaying by PCR. The type strains of bacteria used were *C. difficile* strain ATCC 9689, and *C. sordellii* strains ATCC 9714 and CDC-14337. *C. difficile* strain 630 was obtained from Dr H. Hachler, University of Zurich, Switzerland. All other *C. difficile* strains, and one strain of *C. sordellii*, were clinical isolates from Heidelberg Repatriation Hospital, Heidelberg, Australia.

Culture method

C. difficile was isolated from human stools by inoculation on to *C. difficile* Agar (Oxoid), supplemented with cycloserine 125 mg/L and cefoxitin 4 mg/L (Oxoid), and incubation at 36°C in an anaerobic atmosphere for 48 h (T. Riley, personal communication). Identification was confirmed by gas-liquid chromatography and biochemical tests.⁹

Cytotoxin assay

The method used was based on that of Chang *et al.*¹⁰ Briefly, faecal specimens were diluted five-fold in sterile phosphate-buffered saline (PBS), vortex mixed for 10 s, and then centrifuged for 15 min at 900 *g*. The supernate was passed through a 0.2 µm filter (Millipore). Portions (20 µl) of both the neat supernate and a 10-fold dilution (in PBS) were tested for cytotoxicity by their addition to 200 µl of Ham's F10 growth medium (Gibco), supplemented with fetal bovine serum (Gibco) 5%, over a monolayer of BHK cells (ICN Biomedicals, Seven Hills, NSW, Australia) in the wells of a microtitration tray (ICN Biomedicals). Specificity was established by setting up wells with and without 20 µl of a five-fold dilution of *C. sordellii* antitoxin (Wellcome). Microtitration trays were incubated at 37°C in CO₂ 5% for 24 h before reading the results. Rounding of the cells in wells containing supernate only, but not in those containing both supernate and antiserum, constituted a positive result. Cell rounding with neat supernate, but not with the 10-fold dilution, was considered to be a weak positive reaction. The positive control was supernate from a 72-h culture of *C. difficile* in Brain Heart Infusion Broth (Oxoid).

Preparation of samples for PCR assay

A small quantity of faeces (wooden end of swabstick dipped into faecal specimen) was resuspended in 250 µl of sterile H₂O in a 1.5-ml microfuge tube (Kartell, Naviglio, Italy), vortex mixed for 10 s, boiled for 10 min, and then placed on ice. Faecal solids were removed by centrifugation at 24600 *g* for 5 min; the supernate was used as a crude DNA preparation in the PCR assay. For the *C. difficile* strains, two colonies grown on Columbia Agar (Amey Media, Moorabin, Victoria, Australia), supplemented with horse blood (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) 5% v/v, were resuspended in 250 µl of sterile H₂O and processed as above.

DNA extraction by the Qiagen procedure

Faecal material was added to 500 µl of sterile H₂O in a 1.5-ml microfuge tube to give a thick slurry. The suspension was boiled for 10 min, and then centrifuged at 24600 *g* for 10 min to remove faecal solids. The sample was prepared for extraction by adding 420 µl of buffer (1.5 M NaCl, 50 mM 3-[N-morpholino]propane sulphonic acid, pH 7.0) to 180 µl of the supernate. The DNA was extracted with a Qiagen-tip 20 (Diagen, Dusseldorf, Germany) according to the manufacturer's instructions. The final DNA pellet was washed with ethanol 70%, dried for 10 min in a Speedvac concentrator (Savant, Farmingdale, NY, USA), and then redissolved in 50 µl of sterile H₂O.

PCR assay

Each reaction mixture contained either positive

control crude DNA preparation (2 µl), crude faecal DNA preparations (2 µl or 10 µl), or Qiagen extracts (2 µl or 10 µl) in a total reaction volume of 100 µl with 5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 5 mM KCl, gelatin 0.01 % w/v, Triton X100 0.1 % v/v, 200 µM of each deoxyribonucleotide, 50 pM each of primers BW69 (GAA GCA GCT ACT GGA TGG CA) and BW70 (AGC AGT GTT AGT ATT AAA GT) (Bresatec, Adelaide, Australia), and *Taq* polymerase (Boehringer Mannheim) 4 U. Procedures for amplification and analysis were those of Wren *et al.*⁷ Further analysis of DNA bands separated by electrophoresis was performed with Bio Image Software (Millipore).

Results

Band patterns after PCR of cultures of *C. difficile* isolates

After DNA extraction and PCR, the *C. difficile* positive control, strain ATCC 9689, generated a DNA ladder comprising bands that were 65, 128, 190, 264,

Table. Results of culture, cytotoxin and PCR assays performed with stool samples "positive" for *C. difficile*

Patient	Specimen no.	Culture	Cytotoxin	PCR-1	PCR-2
A	1	—	++	—	+
	2	+	++	+	...
	3	+	++	...	...
	4	+	+	—	+
B	1	+	+	—	+
	2	—	++	+	...
	3	—	+	+	...
C	1	—	++	+	...
	2	—	+	+	...
D	1	+	++	+	...
	2	+	++	+	...
E	1	—	+	—	+
F	1	+	++	—	+
	2	+	++	+	...
	3	—	++	+	...
	4	—	+	—	+
	5	—	+/-	—	+
	6	+	++	+	+
	7	+	++	+	...
	8	+	++	+	...
G	1	—	++	—	+
H	1	—	+	+	...
	2	—	++	—	+
	3	—	+/-	+	...
	4	—	+	+	...
I	1	—	++	—	+
J	1	—	++	+	+
	2	—	++	+	+
	3	+	—	—	...
K	1	+	++	+	...
	2	—	++	+	...
L	1	+	++	+	...
	2	—	++	+	+
M	1	+	++	—	...
	2	—	++	+	...
N	1	—	++	+	...
O	1	—	+	+	...

PCR-1, PCR assay with DNA extraction by simple boiling; PCR-2, PCR assay with DNA extraction by the Qiagen procedure.

336, 417 and 502 bp in size. The nine *C. difficile* clinical isolates, together with strains 630 and ATCC 9689 (all of which were cytotoxin-positive), generated either one (65 bp) or more of these bands after PCR, whereas the five clinical isolates that were cytotoxin-negative did not produce any PCR products corresponding to the characteristic ladder. Three clinical isolates generated atypical band patterns which did not correspond to those of *C. difficile*; these results were considered to be negative. The band patterns produced by three strains of *C. sordellii* did not correspond to those of *C. difficile*.

Comparison of methods for direct examination of stool samples

Cytotoxin assay. From a total of 172 specimens obtained from 58 patients between November 1990 and September 1991, 37 gave positive results by culture or cytotoxin assay for *C. difficile*. Of the 37 "positive" stool specimens, 36 (97%) were cytotoxic (table), of which 25 gave strongly positive results, nine gave weakly positive results and two produced only a very weak response (partial rounding with the neat filtrate).

Culture method. Only 15 (41%) of the 37 "positive" stools gave a positive culture result. One of the 37 samples was culture-positive, but cytotoxin-negative (table).

PCR assay. Of the 37 "positive" stools, it was possible to test 36 by the PCR assay. Of these, 24 gave a positive result by direct testing, 10 gave positive results after Qiagen extraction, and in the remaining two instances (samples J3 and M1; table) there was insufficient specimen to perform the Qiagen extraction procedure. Qiagen extraction was also performed on four specimens which were PCR-positive by the boiling method; in all four cases the result was enhanced by the appearance of additional bands.

Of the stool samples that gave negative results for *C. difficile* by culture and cytotoxin testing, 21 were selected and tested by the direct PCR assay (i.e., without Qiagen extraction); one sample was PCR-positive and 20 were PCR-negative, including seven samples that generated atypical band(s).

Discussion

The PCR method for detection of a segment of the *C. difficile* enterotoxin gene directly from stool samples was more rapid than the culture method, and of sensitivity similar to that of the cytotoxin assay. Of importance to the routine laboratory, the PCR assay can be completed within 3 h, whereas the culture method requires at least 2 days for a preliminary result and cytotoxin results require 24 h, although results are sometimes obtained after 4 h for strongly positive specimens.

The presence of removable PCR inhibitors has been demonstrated in urine.¹¹ The decrease in PCR pro-

ducts found in larger volume samples suggests that inhibitors may be present also in faeces. Whereas urine inhibitors can be removed by molecular exclusion filtration, this procedure was not effective in the present study (results not shown). DNA purification by the Qiagen extraction procedure increased the level of detection, thereby making it comparable to the cytotoxin assay. However, use of the Qiagen procedure adds another step and extra cost; alternative methods for cleaning and concentrating the specimen are being investigated. During the course of this investigation, specimens underwent freezing and thawing. This may have decreased the sensitivity of the assay.

In several instances, faeces that gave negative results in the cytotoxin assay generated one or more PCR products of different size to those of the typical *C. difficile* ladder. A possible explanation for this is that some other bacterium present in the faeces had similar DNA sequences. The emergence of non-toxigenic strains from mice challenged with toxigenic strains¹² suggests that re-arrangement of the genome of *C. difficile* or another clostridial species might occur. This phenomenon was not investigated further here; however, the patterns obtained did not match those generated by PCR with three *C. sordellii* strains. Wren *et al.* showed that 23 other clostridial species, tested with the same primers, did not produce any PCR products.¹³

Several methods employing PCR for the identification of *C. difficile* have been developed recently.¹⁴⁻¹⁶ Our method was based on that of Wren *et al.*,⁷ and employs primers that recognise a repetitive region of the enterotoxin gene to generate a distinct ladder of amplification products.¹⁷ Recent reports have demonstrated the direct detection of infectious agents, including *C. difficile*, in stool samples.^{16,18,19} However, all of these methods involve DNA extraction procedures which would be difficult and time-consuming to perform on a large scale. The assay reported here is rapid and easy to use, similar in sensitivity to the cytotoxin test, specific in its detection of the enterotoxin gene, and has the potential to be used in the routine laboratory.

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