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Detection of *Clostridium difficile* toxins by enzyme immunoassay

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SUMMARY

An enzyme-linked immunosorbent assay (ELISA) for the rapid diagnosis of antibiotic-associated colitis (AAC) is presented. Commercially available antisera to *Clostridium difficile* toxins contain antibodies to other antigens found in non-toxigenic *C. difficile* and other bacteria. Removal of these unwanted antibodies by absorption increased the specificity of ELISA for detection of *C. difficile* toxins. Specimens tested included 40 faecal extracts positive for cytotoxicity from cases of AAC, 30 diarrhoeic and 30 well-formed stools negative for cytotoxicity and 50 culture filtrates of toxigenic and non-toxigenic *C. difficile* and other clostridial species. Use of absorbed sera reduced false-positive reactions observed with faecal specimens from 23 to 8 %. About 90 % of specimens that were positive by the tissue culture cytotoxicity test were positive by ELISA using the absorbed sera. The relative merits of ELISA and other methods for the rapid diagnosis of AAC are discussed.

INTRODUCTION

Antibiotic-associated colitis (AAC) and diarrhoea are being reported in increasing numbers as complications of antibiotic therapy (George, 1984). The need for a rapid and reliable laboratory method for the diagnosis of AAC or pseudomembranous colitis has been felt for several years. Tissue culture facilities essential for carrying out the diagnostic cytotoxicity assays are not available in most laboratories (Bartlett, 1981). Moreover cytotoxicity tests and confirmatory neutralization tests require 48–72 h. Selective media are available for anaerobic culture but this also takes 48 h or more for growth and identification of *C. difficile* and does not differentiate between toxigenic and non-toxigenic strains of *C. difficile* (Bartlett, 1981; George, 1984). The availability of specific therapy in the form of oral vancomycin or metronidazole has also increased the need for rapid aetiologic diagnosis of AAC (Bartlett, 1984).

Detection of toxin in faeces by immunological means has been reported by many investigators. Counterimmunoelectrophoresis (CIE) has been the most widely used method (Ryan, Kwasnik & Tilton, 1980; Welch, Menge & Matsen, 1980; Levine, Kennedy & Lamont, 1982; Wu & Fung, 1983; Jarvis *et al.* 1983; Rennie *et al.* 1984; Lyerly, Phelps & Wilkins, 1985). Latex agglutination (Shahrabadi *et al.* 1984; Lyerly, Phelps & Wilkins 1985) and ELISA (Yolken *et al.* 1981; Lyerly, Sullivan & Wilkins 1983; Laughon *et al.* 1984) have not been as widely evaluated. The time

of obtaining results from these techniques varies from 5 min to 4 h. ELISA appears to be the most sensitive of these being able to detect very small quantities of toxin (Laughon *et al.* 1984; Lyerly, Sullivan & Wilkins, 1983). Most laboratories have facilities to perform ELISA and results are available in about 4 h. However, suitable antisera are not commercially available for performing ELISA or CIE and published results indicate a lack of specificity in most laboratories. Antisera that are commercially available are produced against toxins of *C. difficile* or against toxins of *C. sordellii*. All these antisera contain antibodies to many antigens besides antitoxins to toxins A and B of *C. difficile* (Poxton & Byrne, 1981; West & Wilkins, 1982; Ryan, Kwasnik & Tilton, 1983; Laughon *et al.* 1984; Rennie *et al.* 1984). The specificity of ELISA was enhanced by using absorbed sera as reported here.

MATERIALS AND METHODS

Faecal specimens

Solid and semi-solid stool samples were diluted 1:4 (wt/vol.) with phosphate buffered saline (PBS). Liquid and watery stools were diluted with an equal volume of PBS. After thorough mixing they were centrifuged at 12000 ***g*** and the supernatant fractions filter-sterilized with a 0·22 μm filter and stored at −20 °C.

Bacterial cultures

Clostridia used in the study included 22 strains of non-toxigenic *C. difficile*, 2 strains each of *C. bifermentans*, *C. novyi*, *C. perfringens*, *C. ramosum* and *C. sporogenes* and 18 strains of toxigenic *C. difficile*. All of the above were isolated in our laboratory and identified by bacteriological methods including gas liquid chromatography and by the API 20A anaerobe identification kit (API Laboratory Products Ltd, Saint-Laurent, Quebec, Canada). Pre-reduced anaerobically sterilized chopped meat glucose broth (CMG) (10 ml tubes) were used for growing all the clostridia included in the study. Cultures were grown for 3 days and the bacterial suspensions were centrifuged at 12 000 ***g***. Supernatant fractions were sterilized by filtration through a 0·22 μm filter and stored at −20 °C. The bacterial pellets were used for absorbing sera as described below.

Toxin preparations

Toxin preparations were made from a standard strain of *C. difficile* (VPI no. 10463, Virginia Polytechnic Institute, Blacksburg, Virginia, USA). The strain was cultured in dialysis tubing according to the method of Ehrich *et al.* (1980). Modifications in the purification process by Bloomberg (1984) included precipitation of culture filtrate proteins with 70 % ammonium sulphate followed by gel filtration and ion exchange chromatography with an NaCl gradient. The mixed A and B toxin fractions had a specific cytotoxic titre of 10^8 at a protein concentration of 1 mg/ml.

Antisera

Commercially available rabbit and goat antisera to *C. difficile* toxins were obtained from Virginia Polytechnic Institute, Blacksburg, Virginia, USA. These antisera were chosen instead of antiserum to *C. sordellii* toxin because (*a*) the antibody is prepared against homologous toxins and therefore less likely to contain

cross-reactive antibodies and (*b*) they have a much higher titre for *C. difficile* toxins than *C. sordellii* antitoxin (Ehrich *et al.* 1980).

Tissue culture cytotoxicity assay

Tenfold serial dilutions of faecal specimens and culture filtrates were made in PBS and tested on monolayers of McCoy cells in 96-well plates. Results were read at 24 and 48 h and were considered positive where 50 % or more of the cells showed cytopathic change. Confirmatory neutralization tests were done using goat antiserum to *C. difficile* toxins at a dilution of 1:50.

Absorption of sera

Supernatant fractions of 22 strains of non-toxigenic *C. difficile* were tested against unabsorbed rabbit and goat antisera. Two that gave maximum reactivity in the ELISA were chosen. Also one strain each of *C. bifermentans*, *C. novyi*, *C. perfringens*, *C. ramosum* and *C. sporogenes* were used. All cultures were grown in CMG for 3 days. Bacterial pellets from 20 ml of CMG were washed three times in saline. Each antiserum was diluted 1:10 in saline containing sodium azide (0·02 %) and mixed with the pooled bacteria. After incubation with shaking at 37 °C for 2 h they were kept at 4 °C overnight. Tubes were centrifuged at 12000 ***g*** and the supernatant fractions saved. In order to remove antibodies to soluble antigens in CMG supernatant fractions the following additional step was employed. Nitrocellulose and Zeta-Probe (TM) blotting membranes (Bio-Rad Laboratories, Richmond, California, USA) were finely shredded and added to the pooled CMG supernatant fractions. The binding of soluble proteins to the membrane fragments was allowed to take place for 4 h at 37 °C and for 18 h at 4 °C. The membranes were washed three times in saline. Antiserum previously absorbed with pooled bacterial cells was added to the membranes and incubated at 37 °C for 2 h and overnight at 4 °C. The suspension was then centrifuged at 12000 ***g*** and filter-sterilized with 0·22 μm filter.

Enzyme-linked immunosorbent assay

Indirect or double-sandwich ELISA was done following the method of Voller, Bidwell & Bartlett (1980) with some modifications. Flat-bottomed 96-well polystyrene microtitre plates (Immulon II – Dynatech Laboratories, Alexandria, Virginia, USA) were coated with 0·3 ml of a 1:500 dilution of either rabbit or goat antiserum (absorbed or unabsorbed) in carbonate buffer (pH 9·6). The plates were sealed with Parafilm (American Can Corp., Greenwich, Connecticut, USA). After incubation overnight at 4 °C the plates were washed three times in PBS containing 0·05 % Tween 20, pH 7·4 (PBST). Sealed plates could also be stored at 4 °C for up to 4 weeks if necessary without decrease in reactivity. Foetal bovine serum, 0·1 ml was added to all the wells to prevent desorption of coated protein (Viscidi *et al.* 1984). An equal volume of culture or faecal filtrate was then added to duplicate wells to give a final volume of 0·2 ml. Controls included PBS, CMG and known toxin preparations. All subsequent washes were done five times in PBST and incubation periods were for 1 h at 37 °C except the final incubation following addition of substrate. Plates were sealed with Parafilm during all incubation periods. The second antiserum (detecting antibody) was used at a dilution of 1:500 in PBST. If an absorbed antiserum was used in coating plates then an absorbed antiserum

was also used as the second antibody. Thus the pair of rabbit and goat antisera employed was matched as both absorbed or both unabsorbed sera. Appropriate enzyme conjugates (alkaline phosphatase) were used depending on the species of the detecting antibody (Sigma Chemical Co., St Louis, Missouri, USA). Para-nitrophenyl phosphate (Sigma Chemical Co.) was used as the substrate at a concentration of 1 mg/ml of diethanolamine buffer (pH 9·8). Substrate was added in a volume of 0·2 ml and incubated for 30 min at room temperature in the dark. The reaction was brought to a stop by the addition of 0·05 ml of 3 N-NaOH. Absorbance was measured at 410 nm with a microplate reader (Minireader II – Dynatech Laboratories). All positive specimens were tested a second time with antisera and with non-immune rabbit or goat sera. A base line absorbance for faecal specimens was arrived at by calculating the mean absorbance of 30 lower results (50 %) of the 60 specimens tested from patients who did not have AAC. A positive result was defined as an absorbance that exceeded the above mean value by two standard deviations or more. The base line absorbance for culture filtrates was that produced by uninoculated CMG medium.

RESULTS

It was observed during preliminary experiments in the ELISA procedure that rabbit and goat antisera had to be paired as both absorbed or both unabsorbed. The use of an unabsorbed antiserum in one step with an absorbed serum in the other step resulted in almost as many false-positive reactions as that using unabsorbed sera in both steps. This may be due to the presence of soluble antigen-antibody complexes in the absorbed antiserum that react with antibodies in the unabsorbed antiserum.

The additional step of using nitrocellulose and Zeta-Probe (TM) blotting membranes for absorbing antisera resulted in much improved specificity. The antitoxins are prepared by injecting animals with a crude culture filtrate of toxigenic *C. difficile* and it is conceivable that antibodies to bacterial cellular and extracellular products may be present in the antisera (Ehrich *et al.* 1980). ELISA tests were done in parallel using antisera absorbed with bacterial cells only and antisera absorbed with bacterial cells and membrane suspension with protein bound to it. Ten CMG supernatant fractions of non-toxigenic *C. difficile* and other clostridial species were tested and the reactivity was found to be greatly decreased or abolished where the antiserum that had been absorbed additionally with blotting membrane was used. Reactivity with toxigenic strains of *C. difficile* was not significantly affected by such absorption procedures.

Results of culture supernatant fractions using rabbit antiserum for coating plates show that the unabsorbed serum gave positive reactions with all 22 strains of non-toxigenic *C. difficile* and 5 out of 10 other clostridial strains. Absorbance was higher in the case of non-toxigenic *C. difficile* and *C. bifermentans* and lower with *C. perfringens* and one strain of *C. novyi*. Two strains each of *C. ramosum* and *C. sporogenes* and one strain of *C. novyi* were negative. The absorbed antisera reacted only with 2 strains of non-toxigenic *C. difficile* though it retained a high level of reactivity against 18 strains of toxigenic *C. difficile* and purified toxin preparations. The results are statistically significant with a chi-square value of 36 ($P < 0{\cdot}001$).

Table 1. *ELISA results of tissue culture toxicity negative clostridial strains* (C. bifermentans, C. perfringens, C. novyi, C. ramosum *and* C. sporogenes (*2 strains each*); C. difficile *non-toxigenic strains 22, n = 32*). *Rabbit antiserum used for coating plates*

Absorbed rabbit serum	Unabsorbed rabbit serum		
	Positive	Negative	Total
Positive	2	0	2
Negative	25	5	30
Total	27	5	32

Table 2. *ELISA results of tissue culture toxicity negative faecal specimens* (*culture negative for toxigenic* C. difficile, *n = 60*). *Rabbit antiserum used for coating plates*

Absorbed rabbit serum	Unabsorbed rabbit serum		
	Positive	Negative	Total
Positive	8	0	8
Negative	10	42	52
Total	18	42	60

Table 3. *ELISA results of tissue culture toxicity negative faecal specimens* (*culture negative for toxigenic* C. difficile, *n = 60*). *Goat antiserum used for coating plates*

Absorbed goat serum	Unabsorbed goat serum		
	Positive	Negative	Total
Positive	5	0	5
Negative	9	46	55
Total	14	46	60

Many faecal specimens from patients who did not have AAC and which were negative for tissue culture cytotoxicity and negative on culture for *C. difficile* gave positive reactions with unabsorbed antisera. Though positive, the absorbance readings were lower than those obtained with toxin containing faecal specimens. Eighteen of 60 faecal filtrates were positive with unabsorbed rabbit antiserum used as first antibody and 14 were positive with unabsorbed goat antiserum as first antibody. After absorption 8 positive readings were observed with rabbit antiserum and 5 positive readings with goat antiserum. The difference between using absorbed and unabsorbed antisera for tissue culture negative faecal specimens was statistically significant ($\chi^2 = 4$; $P < 0{\cdot}05$). Non-toxigenic *C. difficile* strains were grown from 5 out of 60 faecal specimens. All 5 were positive by ELISA using unabsorbed sera whereas only 2 of 5 were positive with absorbed sera. Cultures for other clostridial species were not done. Moderately high absorbancy readings were also noted when faeces contained *Staphylococcus aureus*. This occurred when rabbit antiserum was used as the first or coating antibody and was probably due to staphylococcal A protein. This type of false reactivity was detected by the parallel use of non-immune rabbit serum which also gave high readings with staphylococcal A protein. Goat serum used as first antibody did not give rise to this difficulty as staphylococcal A protein does not bind to goat immunoglobulins.

Table 4. *ELISA results of 40 faecal specimens from patients with AAC; positive for tissue culture toxicity and positive for isolation of toxigenic* C. difficile. *Rabbit antiserum used for coating plates*

Absorbed rabbit serum	Unabsorbed rabbit serum		
	Positive	Negative	Total
Positive	37	0	37
Negative	1	2	3
Total	38	2	40

Table 5. *ELISA results of 40 faecal specimens from patients with AAC; positive for tissue culture toxicity and positive for isolation of toxigenic* C. difficile. *Goat antiserum used for coating plates*

Absorbed goat serum	Unabsorbed goat serum		
	Positive	Negative	Total
Positive	35	0	35
Negative	2	3	5
Total	37	3	40

Faecal specimens from patients with AAC that were positive for tissue culture cytotoxicity and from which toxigenic *C. difficile* had been isolated in culture produced high absorbancy readings in ELISA. Absorbed rabbit antiserum and absorbed goat antiserum used as first antibody were able to detect toxins in 92·5 and 87·5 % of specimens respectively (Tables 4 and 5). All faecal filtrates that had a cytotoxicity titre of 10^3 or more were positive by ELISA. Five specimens that were negative with ELISA (Table 5) had a titre of 10^2 or less in the tissue culture assay. Statistically no significant difference could be demonstrated between the use of absorbed and unabsorbed sera or between rabbit and goat antisera with specimens that were positive by culture and by cytotoxicity assay.

DISCUSSION

All cases of diarrhoea and colitis associated with antibiotic therapy are not caused by *C. difficile*. A large proportion of sporadic cases of enterocolitis caused by *C. difficile* goes undiagnosed aetiologically for the lack of a reliable and easily available laboratory test. The discontinuation of the offending antibiotic therapy and specific treatment with oral vancomycin or metronidazole are essential steps in the management of more serious cases of *C. difficile*-induced AAC (George, 1984; Bartlett, 1981; Bartlett, 1984). These observations have made an aetiologic diagnosis of AAC important for the hospitalized patient. Most hospitals do not have facilities for tissue culture work for performing the highly specific and sensitive cytotoxicity assays (Laughon *et al.* 1984). There is clearly a need for a rapid and reliable laboratory test for the diagnosis of AAC. Immunodiagnostic tests (ELISA and CIE) have been described but interpretation of results has been difficult due to the presence of non-specific antibodies in available antisera. A large number of false-positive results are likely because of the presence of antibodies to non-toxigenic *C. difficile* and other clostridia (Yolken *et al.* 1981; Poxton & Byrne, 1981; West

& Wilkins, 1982). Enzyme immunoassays using affinity-purified antibodies to toxins A and B have been described (Lyerly, Sullivan & Wilkins, 1983; Laughon *et al.* 1984). The sensitivity of detection of toxin B by ELISA is reported to be less than that of the cytotoxicity assay (Laughon *et al.* 1984). This is understandable in view of the exquisite sensitivity of mammalian cells to toxin B. However detection of toxin A by ELISA is much more sensitive than tissue culture assay because of the weak cytotoxic action of toxin A (Lyerly, Sullivan & Wilkins, 1983). The proportion of these toxins produced by *C. difficile* in vivo is not well established. In a clinical setting an ELISA test using reagents directed against both toxins is likely to be more sensitive than ELISA that detects only one toxin.

In our laboratory the use of unasorbed antisera for ELISA produced 23 % false positive results which is an unacceptably large number. Absorption of antisera with various clostridia reduced this to 8 %. This is an acceptable level of specificity for a screening test which has to be interpreted after taking into account the clinical features of the patient. The sensitivity of the ELISA was not diminished significantly after absorption. Of the tissue culture positive specimens 87·5 % were positive in the ELISA test using goat antiserum as first antibody. All faecal extracts which had a cytotoxicity titre of 10^3 or more gave a positive reaction in ELISA. The sensitivity of ELISA correlated well with results of tissue culture assay and are similar to those described by Lyerly, Sullivan & Wilkins (1983).

The ELISA test described here takes about 4 h to perform if previously coated plates are available. This is a considerable saving of time when compared with the time required for a cytotoxicity assay or anaerobic culture on selective media. Of the other immunodiagnostic tests CIE results may be available in 1–2 h and latex agglutination in 5–15 min (Shahrabadi *et al.* 1984; Lyerly, Phelps & Wilkins, 1985). However the level of sensitivity of these rapid tests appears to be much less than that of ELISA and there appears to be considerable disagreement about the utility of CIE as a diagnostic test (Poxton & Byrne, 1981; West & Wilkins, 1982). In the case of CIE use of absorbed sera is reported to increase specificity (Ryan, Kwasnik & Tilton, 1983; Rennie *et al.* 1984). Use of monoclonal antibodies for CIE, latex agglutination and ELISA has recently been described (Lyerly, Phelps & Wilkins, 1985). Of these ELISA is said to be the most sensitive. In our laboratory we are in the process of producing monoclonal antibodies to *C. difficile* toxins.

Positive reactions in ELISA with clostridia were largely abolished by absorption though some strains of non-toxigenic *C. difficile* reacted even with the absorbed sera. The difficulty of completely removing cross-reactive antibodies by absorption has been reported by Laughon *et al.* (1984) and Shahrabadi *et al.* (1984). The technique described in this report is inexpensive and within the reach of most hospital laboratories and removes most of the unwanted antibodies to cellular and extracellular antigens of clostridia. Until monoclonal antibodies or affinity-purified antibodies of high specificity and sensitivity to *C. difficile* toxins become widely available ELISA using absorbed antisera may be regarded as a valid test for the rapid diagnosis of AAC caused by *C. difficile*.

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