

Enzyme immunoassay (ELISA) for detection of *Clostridium difficile* toxin B in specimens of faeces

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Summary. Antisera against *Clostridium difficile* toxin B were prepared in sheep and rabbit and were used in indirect and sandwich enzyme-linked immunosorbent assays (ELISA) for the detection of toxin B. Polyvinyl chloride and polystyrene microtitration plates were tested as solid phases for the assay. Both assays had a lower limit of detection for toxin B of 1 ng/ml. They were used to detect the presence of toxin B in 210 human faecal specimens and also in the culture supernatant fluids of *C. difficile* strains isolated from the faecal samples. There was a close correlation between the results of sandwich ELISA and those of cytotoxicity tests and isolation of *C. difficile*. Our sandwich ELISA method seems to be useful as a presumptive test for detection of *C. difficile* toxin B.

Introduction

Clostridium difficile, the major cause of antibiotic-associated colitis and diarrhoea in man, produces two toxins; toxin A causes fluid accumulation in rabbit ileal loops and has been called an “enterotoxin”, and toxin B has been described as primarily a cytotoxin (Banno *et al.*, 1981; Taylor *et al.*, 1981). Because of the severity of the disease in many cases, several methods for detecting *C. difficile* (George *et al.*, 1979; Borriello and Honour, 1981) or its toxins (Ehrich *et al.*, 1980) have been devised. However, these methods are time consuming and not well suited for routine use in clinical laboratories. Recently, counter-immuno-electrophoresis (CIE) (Levine *et al.*, 1982), latex agglutination (Shahra-badi *et al.*, 1984) and enzyme-linked immunosorbent assay (ELISA) (Yolken *et al.*, 1981; Krishnan, 1986) have been used to detect *C. difficile* toxin in faecal and culture filtrates, but the results obtained are not entirely satisfactory and their routine use in clinical laboratories is, therefore, restricted.

The purpose of the present study was to investigate methods for the rapid diagnosis of disease associated with *C. difficile* by detection of *C. difficile* toxin B in faecal and culture filtrates by an ELISA. Various solid-phase supports (microtitration plates) were tested in indirect and sandwich ELISA methods. Results obtained by ELISA were compared with isolation of *C. difficile* and assay for cytotoxicity in McCoy cells.

Materials and methods

Faecal specimens

Solid and semi-solid samples of faeces were diluted 1 in 10 (w/v) with phosphate-buffered saline (PBS) (0.05 M, pH 7.4). After thorough mixing, they were centrifuged (12 000 *g* for 30 min) and the supernates were sterilised by filtration through a 0.22- μ m filter (Sartorius, 91122 Palaiseau Cedex, France). These faecal filtrates were then assayed for cytotoxicity on monolayers of McCoy cells.

Culture of *C. difficile*

Heavy inocula of faecal specimens were made on to Cycloserine-Cefoxitin-Fructose Agar, (George *et al.*, 1979). Plates were incubated anaerobically at 37°C and examined after 48–72 h for characteristic colonies of *C. difficile*. The *C. difficile* isolates were then grown anaerobically in Peptone-Yeast extract-Glucose (PYG) medium (Dowell and Hawkins, 1974) for 72 h at 37°C to test for cytotoxic activity. After centrifugation, the culture supernate was sterilised by filtration through a 0.45- μ m filter and assayed for cytotoxicity on monolayers of McCoy cells.

Preparation of toxin B

Toxin B was prepared in our laboratory as described previously (Rihn *et al.*, 1988).

Chromatographic analyses were performed on a fast protein liquid chromatography (FPLC) apparatus (Pharmacia, Uppsala, Sweden). Protein content was measured by the method of Bradford (1976) by reference to a calibration line obtained with bovine serum albumin

(BSA) (IBF Biotechnics, Villeneuve-la-Garenne, France) as a standard.

Preparation of antisera

A 3-kg rabbit was given 0.1 mg of purified toxin B (inactivated by formaldehyde 0.4% in PBS) and an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) by subcutaneous injection. Injections were repeated twice at intervals of 15 days. The rabbit was bled 5 days after the third injection and the serum was tested for neutralising activity. The same procedure was used for immunisation of sheep. Each animal received 0.5 mg of purified toxin B as used for immunisation of the rabbit. All antisera were treated by selective precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 40% saturation for isolation of IgG (Garvey *et al.*, 1977). The IgG fraction obtained was further purified by chromatography on a Mono Q anion exchange column (100×10 mm, Pharmacia, Uppsala, Sweden). Elution was performed with an NaCl gradient in 0.01 M Tris HCl buffer, pH 7.4.

Cytotoxicity and neutralisation assays

The cytotoxicity of *C. difficile* toxin was assayed with monolayer cultures of McCoy cells maintained in Minimal Essential Medium (MEM) (Seromed, Berlin, FRG) supplemented with L-glutamine 5% and fetal calf serum 5% in a 96-well microtitration plate (Tytgat, 1980). Serial two- and ten-fold dilutions of culture or faecal filtrate were made in MEM and tested on monolayers of McCoy cells; 0.02 ml of each dilution was added to the wells. The microtitration plate was incubated at 37°C in air + CO₂ 5%. The endpoint of cytotoxicity was defined as the highest dilution of the filtrate that caused rounding of all the McCoy cells after incubation for 24 h.

Neutralisation was determined by pre-incubation of serial dilutions of culture or faecal filtrate with an equal volume of a 1 in 10 dilution of rabbit or sheep antiserum against *C. difficile* toxin B for 20 min at room temperature, followed by cytotoxicity assay. Cells were examined for cytotoxicity after incubation for 24 h.

Enzyme-linked immunosorbent assays (ELISA)

Polyvinyl chloride (PVC-ST, Dutscher, Brumath, France) and polystyrene (Microtest Immuno 96 F, Nunc) 96-well, U bottom microtitration plates were used as the solid phase for the assays. The wells on the outer perimeter gave inconsistent results and elevated background readings and were not used in the assay. A total reaction volume of 0.15 ml was used in all microtitration wells and each experiment was set up in duplicate. All washes were performed four times with PBS containing Tween® 20 (PBS-T) 0.05%. The substrate solution for peroxidase, containing 10^{-2} M *o*-phenylene diamine (Sigma) and 10^{-3} M H₂O₂ was prepared in citrate phosphate buffer (0.1 M, pH 5.5). After incubation for 30 min at room temperature, the absorbance was measured

at 450 nm in a microplate colorimeter (Titertek Multiskan® MC, Flow Laboratories). Results are expressed as optical density (OD) units.

For the indirect ELISA, wells of the microtitration plates were coated with toxin B in sodium carbonate buffer (0.05 M, pH 9.6) or *C. difficile* culture or faecal filtrate. After incubation overnight at 4°C, the plates were washed and a solution of BSA 1% in PBS was added to the wells. The plates were incubated for 1 h at 37°C, washed, and a 1 in 1000 dilution of rabbit anti-toxin B in PBS containing BSA 0.2% (PBS-0.2% BSA) was added. After incubation for 1 h at 37°C, the plates were washed again and a 1 in 2000 dilution of horseradish peroxidase-conjugated affinity chromatography purified goat anti-rabbit IgG (Miles Laboratories Miles, 75755 Paris Cedex 15, France) in PBS-0.2% BSA was added. After incubation again for 1 h at 37°C, the plates were washed and substrate solution was added.

For the sandwich ELISA, wells of microtitration plates were coated with sheep anti-toxin B diluted 1 in 200 in sodium carbonate buffer. After incubation overnight at 4°C, the plates were washed. The coated plates were usually used immediately but could be stored at 4°C for up to 4 weeks without decrease in reactivity. Uncoated attachment sites on the plates were saturated by incubation for 1 h at 37°C with BSA 1% in PBS. The plates were then washed and a solution of toxin B in PBS, alone or in the presence of control (*C. difficile*-negative) faecal filtrate or culture medium or *C. difficile* culture or faecal filtrate was added. After incubation for 1 h at 37°C, the plates were washed again and incubated with the second antibody preparation—a 1 in 1000 dilution of rabbit anti-toxin B in PBS-0.2% BSA. The plates were incubated for 1 h at 37°C and washed before the addition of a 1 in 2000 dilution of peroxidase-labelled goat anti-rabbit IgG in PBS-0.2% BSA. After incubation for 1 h at 37°C, the plates were washed again and the substrate solution was added.

Results

Assay of purified *C. difficile* toxin B

A titration curve was constructed with serial dilutions of purified toxin B preparation of known protein concentration by both indirect and sandwich ELISA procedures with PVC and polystyrene microtitration plates as solid-phase supports. The results (fig. 1) show that for low concentrations of toxin B (1–10 ng/ml), the toxin B curves obtained by the sandwich assay in which the toxin B was immobilised by specific antibody pre-coated on to the well (fig. 1a) gave higher absorbance values than those obtained by the indirect assay in which the toxin B was coated directly on to the well (fig. 1b), whereas similar results were obtained by both methods with higher concentrations of toxin B (100–1000 ng/ml). Lower absorbance values were

obtained in both indirect and sandwich ELISA methods with polystyrene plates than with PVC plates (fig. 1). However, with both types of plate, positive results were obtained with samples containing nanogram concentrations of toxin B. By defining the lower limit of detection as the concentration of toxin B that produced an increase in absorbance value twice as high as the absorbance value of the control wells in which the toxin B was not present, we determined this limit to be 1 ng/ml.

These results showed that the PVC microtitration plates provided the most appropriate solid phase for immobilisation of toxin B and that the sandwich ELISA was the most suitable method for detection of the toxin.

Assay of toxin B in PYG medium or faecal filtrate

Titration curves of toxin B in the presence of faecal filtrate or PYG medium were constructed by indirect and sandwich methods with known amounts of toxin B added to a pooled mixture of faecal filtrates (pH 6.9) from 20 non-cytotoxic and *C. difficile* culture-negative specimens or to PYG medium (pH 6.8). The wells were then coated (in the indirect method) or incubated (in the sandwich method) with these solutions of toxin B. Control wells contained the same solutions without toxin B. The absorbance values obtained were compared with those of the toxin B preparation assayed in the absence of PYG medium or faecal filtrate (see

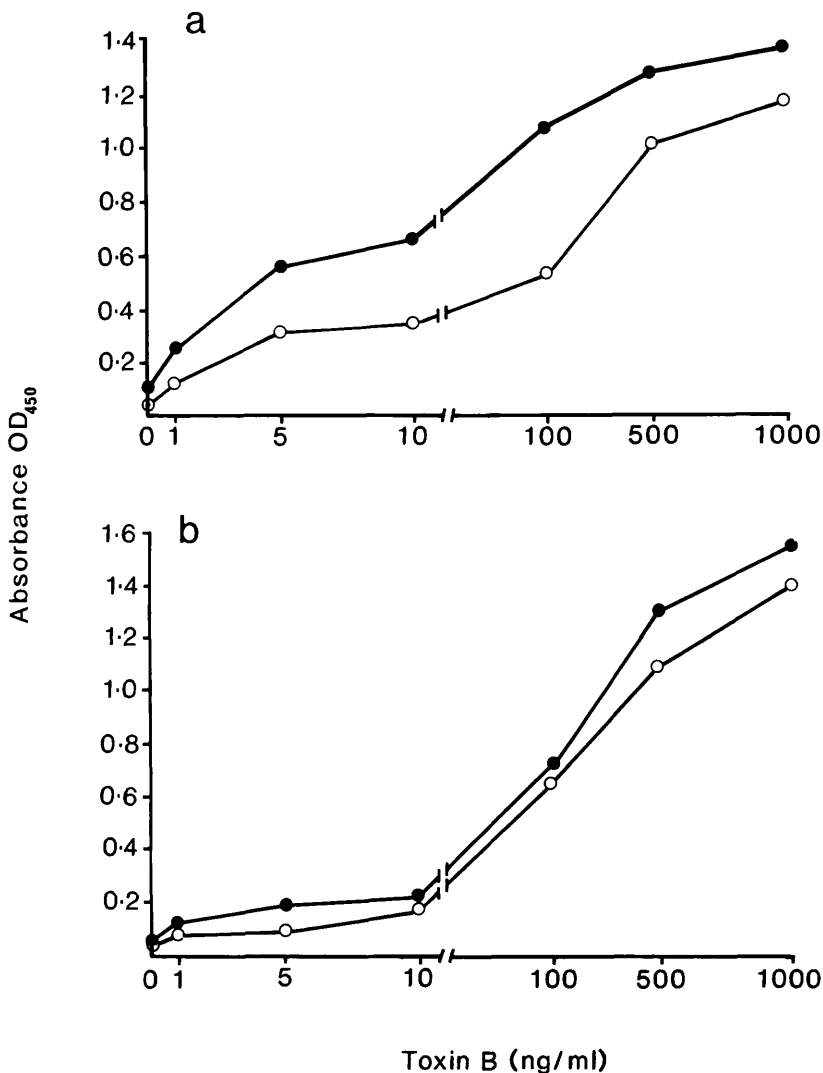


Fig. 1. Titration of toxin B by ELISA: (a) sandwich procedure, (b) indirect procedure in PVC (●) or polystyrene (○) polystyrene microtitration plates. Each point on the curves represents the average value from duplicate samples.

above and fig. 1). The ratio of absorbance values between control and test wells represents the relative sensitivity of detection of toxin B. The results are shown in fig. 2; the control titration curves of toxin B are also reproduced. The presence of PYG fluid or faecal filtrate reduced the sensitivity of detection of toxin B by the indirect ELISA (fig. 2a). However, the presence of PYG medium had no significant effect on detection of toxin B by the sandwich procedure (fig. 2b), although the presence of faecal filtrate reduced the sensitivity by about 60%. The lower limit of detection remained unchanged at 1 ng/ml. We also verified that there was

no significant difference in the titration curves obtained with toxin B by both ELISA methods when the toxin was in PBS (pH 7.4), citrate phosphate buffer (pH 5.5) or sodium carbonate buffer (pH 9.6).

These results suggested that interfering substances in the PYG medium or faecal filtrate limited the immobilisation of toxin B and interfered with the sensitivity of toxin B detection, but attempts to improve the sensitivity of detection of toxin B by diluting the samples in citrate phosphate buffer containing BSA 5% (Viscidi *et al.*, 1984), were unsuccessful.

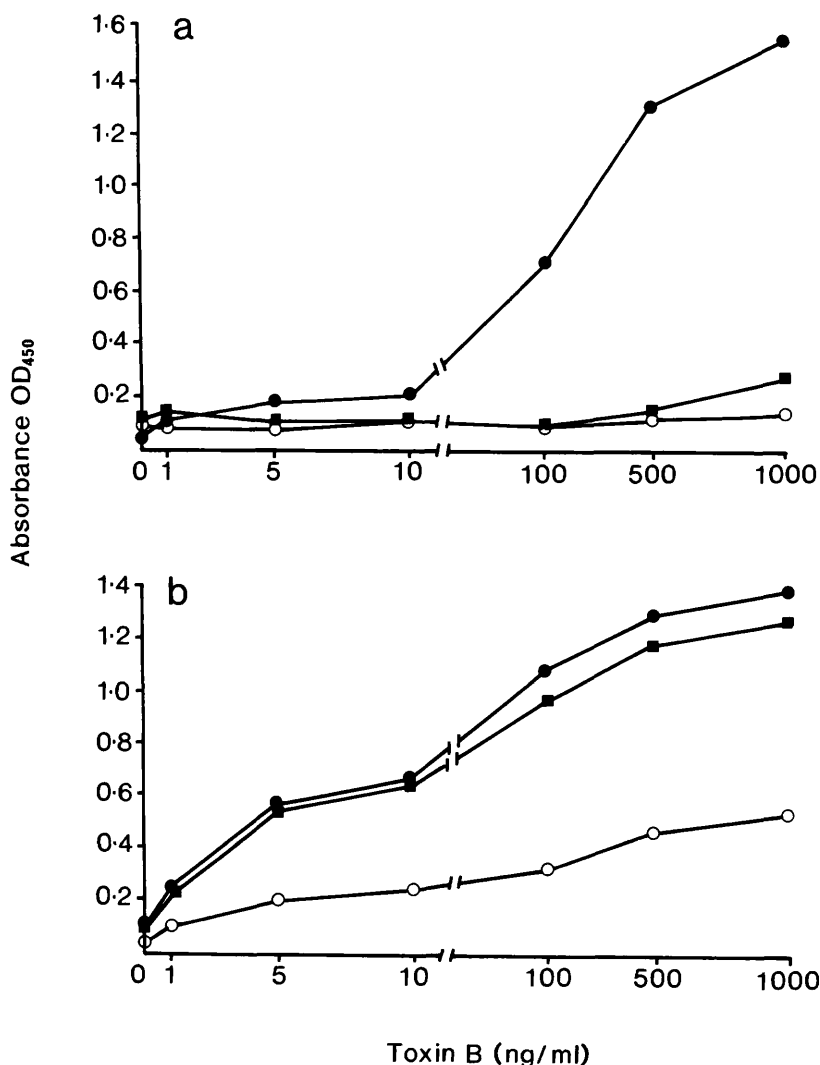


Fig. 2. Titration of toxin B by ELISA in the presence of PYG medium or faecal filtrate by (a) indirect and (b) sandwich procedure; (●) in the presence of (a) sodium carbonate buffer or (b) PBS, (■) in the presence of PYG medium, or (○) in the presence of faecal filtrate. Experiments were performed in PVC microtitration plates and each point on the curves represents the average value from duplicate samples.

Assay of toxin B in clinical samples and culture filtrates

To test the efficacy of our ELISA methods for detecting toxin B in clinical samples, the indirect and sandwich procedures were applied to filtrates of faecal samples and *C. difficile* cultures; the solid phase was PVC microtitration plates.

To examine the correlation of the ELISA results with those of the cytotoxicity assay and isolation of *C. difficile*, 210 faecal specimens from persons suspected of having *C. difficile* colitis were examined. The results are shown in the table. In the 22 specimens that were cytotoxic, the cytotoxicity was neutralised by both sheep and rabbit antisera against *C. difficile* toxin B. Of 186 non-cytotoxic and culture-negative specimens (run 1), only 17 gave positive results by both ELISA procedures (false-positive results, 9%). Two cytotoxic and culture-negative specimens gave positive results by both ELISA procedures (run 3). Of 20 cytotoxic and culture-positive specimens (run 4), 16 (80%) gave positive results by the sandwich ELISA procedure but only 3 (15%) gave positive results by the indirect ELISA method. The faecal filtrates that had higher cytotoxic titres did not always give higher absorbance readings in ELISA. All of the culture supernates tested, despite their lower cytotoxic titres, gave high absorbance readings (strongly positive results) in both ELISA procedures (table).

The absorbance values of faecal and culture filtrates tested by ELISA were not altered significantly after storage at -70°C . No decrease in reactivity was noted for the plates coated with sheep anti-toxin B antibody after storage at 4°C for up to 4 weeks.

Discussion

Solid-phase immunoassays, such as enzyme immunoassays (ELISA) and radioimmunoassays (RIA) are used effectively for the detection of viral, bacterial and toxin-related antigens in faecal specimens. Because antigens might be present in small quantities in the specimen, it is important to maximise the sensitivity of the assay system. In our experimental conditions, both ELISA procedures detected amounts of toxin B as low as 1 ng/ml. However, the presence of faecal filtrate caused an important loss of sensitivity in toxin B detection. The decrease of sensitivity in ELISA systems for the detection of infectious agents in body fluids has been described by several other investigators (Place and Schroeder, 1982; Viscidi *et al.*, 1984). Many

faecal specimens contain an activity, probably proteolytic in nature, which releases the immunoreactant from solid-phase surfaces, thus reducing the sensitivity of ELISA systems for various common faecal antigens. Some of the limitations of ELISA technology were reviewed by Viscidi *et al.* (1984).

In our tests, the sandwich ELISA procedure produced 9% false-positive results which is an acceptable level of specificity for a screening test. Of 20 samples that gave positive results both in the cytotoxicity assay and by isolation of *C. difficile*, 80% also gave positive results in the sandwich ELISA procedure. Thus, there was close correlation between the ELISA results and those of the cytotoxicity test and isolation of *C. difficile*. The 17 (9%) false-positive ELISA results were probably due to cross-reaction of the antitoxins with antigens produced by other non-cytotoxigenic clostridia. The positive results obtained by ELISA for two non-cytotoxic and culture-positive specimens (run 2) could be due to reactions of the antitoxins with antigens produced by non-cytotoxigenic *C. difficile* (Borriello and Honour, 1983). In any case, the presence of such a *C. difficile* strain is exceptional (3%) (George *et al.*, 1978; Tytgat, 1980). The ELISAs and cytotoxic-positive but culture-negative specimens could be due to the presence of only a small number of organisms which could not be detected by the *C. difficile* isolation procedure used (George *et al.*, 1979), which had a lower limit of detection of 2×10^3 organisms/g of faecal sample. The results did indicate that there is not a close correlation between cytotoxic activity and toxin B concentration in the samples. The cytotoxic activity can be affected by various agents and conditions such as temperature, pH, H_2O_2 , O_2 , trypsin, chymotrypsin, pronase, amylase, etc. (Rolfe and Finegold, 1979; Sullivan *et al.*, 1982; Lysterly *et al.*, 1986) without the immunological reactivity of the toxin being altered. However, it should be noted that the concentration of interfering substances present in faecal specimens is one of the main limiting factors in detection of toxin B by ELISA.

Our results are similar to those obtained by ELISA with absorbed antisera for various clostridia described by Krishnan (1986) in which the percentages of false-positive and true positive results were 8 and 87.5% respectively. Use of monoclonal antibodies for CIE, latex agglutination and ELISA has recently been described (Lysterly *et al.*, 1985) and these may further improve the specificity of the test.

However, until monoclonal antibodies to *C. difficile* toxins become widely available, the sand-

Table. Comparison of bacterial isolation, cytotoxicity assay, and ELISA test for detection of *C. difficile* organisms or toxin B in 210 stool specimens

Run	Number of specimens tested	ELISA†													
		Bacterial isolation		Cytotoxicity assay				Indirect method				Sandwich method			
				faecal filtrates		culture filtrates		faecal filtrates		culture filtrates		faecal filtrates		culture filtrates	
		positive	negative	positive (titre)*	negative	positive (titre)*	negative	positive (OD value)	negative (OD value)	positive (OD value)	negative (OD value)	positive (OD value)	negative (OD value)	positive (OD value)	negative (OD value)
1	186	0	186	0	186	NT	NT	17	169	NT	NT	17	169	NT	NT
2	2	2	0	0	2	0	2	2	0	2	0	2	0	2	0
3	2	0	2	2	0	NT	NT	2	0	NT	NT	2	0	NT	NT
	2	2	0	2(10 ²)	0	2(4 × 10 ¹)	0	1(++)	1	2(++)	0	1(++)	1	2(++)	0
	5	5	0	5(2 × 10 ²)	0	5(2 × 10 ¹)	0	1(+)	4	5(++)	0	4(++)	1	5(++)	0
4	7	7	0	7(4 × 10 ²)	0	7(4 × 10 ¹)	0	0	7	7(+++)	0	6(+)	1	7(+++)	0
	3	3	0	3(10 ³)	0	3(8 × 10 ¹)	0	0	3	3(+++)	0	3(+++)	0	3(+++)	0
	3	3	0	3(6 × 10 ³)	0	3(4 × 10 ¹)	0	1(+)	2	3(+++)	0	2(+)	1	3(+++)	0

*The cytotoxic titre is expressed as the reciprocal of the highest dilution of culture or faecal filtrate which causes rounding of 100% of the McCoy cells.

NT, not tested.

†A result was considered to be positive when the OD 450 value was at least twice as high as the value of the negative control (non-cytotoxic and culture-negative specimen); (++) denotes > 1.00 OD unit, (+) denotes 0.4–1.00 OD unit.

wich ELISA procedure seems clinically useful as a presumptive test for the rapid diagnosis of disease associated with *C. difficile*. However, we recommend that positive reactions should be confirmed by both the cytotoxicity test, with neutralisation,

and by the isolation of the organism on a selective medium.

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