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Rapid Detection of *Clostridium perfringens* Type A Enterotoxin by Enzyme-Linked Immunosorbent Assay

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Clostridium perfringens type A enterotoxin was specifically detected and readily quantified by indirect and four-layer sandwich enzyme-linked immunosorbent assays (ELISAs). With the indirect ELISA, enterotoxin was detected in quantities of as low as 2.5 ng (25 ng/ml). When the more sensitive sandwich ELISA procedures was used, 100 pg (1 ng/ml) of enterotoxin was detected. The sandwich ELISA procedure specifically detected enterotoxin in human fecal extracts. Additionally, the sandwich ELISA specifically differentiated enterotoxin-positive strains from enterotoxin-negative strains of *C. perfringens*. Both the indirect and sandwich ELISA procedures described for *C. perfringens* enterotoxin in this report are rapid, specific, sensitive, and easily adaptable for large-scale use by clinical or research laboratories.

Clostridium perfringens type A is one of the most common causes of food-borne disease in the United States (16). A protein enterotoxin which is produced only during sporulation has been shown (18) to be the factor responsible for the characteristic disease symptoms (diarrhea and abdominal cramps) associated with *C. perfringens* food poisoning. *C. perfringens* enterotoxin has been shown (11) to possess a unique plasma membrane mode of action distinguishable from other enterotoxins.

A number of biological and serological assay systems have been developed for detection and quantitation of *C. perfringens* enterotoxin (for review, see reference 12). Currently there is a need for a rapid, simple, and sensitive serological assay for large-scale detection of *C. perfringens* enterotoxin. Enzyme-linked immunosorbent assay (ELISA) methods potentially satisfy these needs, and ELISAs have been developed for the specific detection of several bacterial toxins, including cholera enterotoxin (8), *Escherichia coli* heat-labile enterotoxin (8), *Clostridium botulinum* type G toxin (9), and *Clostridium difficile* toxin A (10).

During the preparation of this manuscript, a four-layer sandwich ELISA was reported (15) for the detection of *C. perfringens* enterotoxin. However, this ELISA procedure requires a minimum test period of 4 days. We now report a rapid sandwich ELISA for the detection of *C. perfringens* enterotoxin which significantly shortens assay time to less than 24 h but retains significant sensitivity and specificity. Additionally, we present a rapid indirect ELISA procedure which appears to be useful for the rapid screening of serum or solutions for antitoxin activity.

MATERIALS AND METHODS

Bacterial strains. *C. perfringens* type A strains NCTC 8239, NCTC 8798, NCTC 10239, FD-1, 8-1, and ATCC 3624 were obtained from James L. McDonel, The Pennsylvania State University, University Park, Pa.

Enterotoxin. Electrophoretically pure enterotoxin was prepared from *C. perfringens* NCTC 8239 (Hobbs serotype 3) by the method of Granum and Whitaker (7).

Immunoglobulins. Rabbit antiserum against purified *C. perfringens* enterotoxin was prepared from New Zealand rabbits. Each rabbit was immunized with 75 μl of antigen solution (2 mg of enterotoxin per ml of saline) in an adjuvant solution prepared with 0.5 ml of Freund complete adjuvant, 0.3 ml of 0.85% saline, and 0.2 ml of Arlacel A (Sigma Chemical Co.). Intradermal injections (30 μl) were given to each rabbit at numerous sites (approximately 40 sites per ml of adjuvant solution). At this immunization only, 0.5 ml of Tri-Immunol (Lederle Laboratories) was injected intramuscularly into each rabbit for immunostimulation. Subcutaneous booster injections of 150 μg of enterotoxin in incomplete adjuvant were given at 2-week intervals. The animals were bled after 8 weeks.

Goat antiserum against highly purified *C. perfringens* enterotoxin was obtained from K. I. Dayalu, The Pennsylvania State University, University Park, Pa.

Specificity of the antisera against enterotoxin was determined by immunoelectrophoresis (6). Immunoelectrophoresis of culture supernatant from strain NCTC 8239 (prepared as described below for screening strains for enterotoxin production) versus either rabbit or goat antienterotoxin yielded a single precipitin line. Additionally, immunoelectrophoresis of purified enterotoxin versus mouse antiserum prepared against NCTC 8239 culture supernatant also yielded a single precipitin line.

ELISA. The ELISA protocol of Voller et al. (22) was used with some modifications.

(i) Indirect ELISA procedure. Samples (100 μl per well) of enterotoxin in phosphate-buffered saline (PBS) were allowed to adsorb to the wells of an Immulon II microtiter plate (Dynatech Laboratories) during overnight incubation in a humid chamber at 4°C. The washing procedure used for both the indirect and sandwich (described below) ELISA procedures involved removing the contents of the wells, filling the wells with 100 μl of warm washing buffer (containing 0.85% NaCl, 0.05% Tween 20, and 0.5% bovine serum albumin) per well, and gently shaking the plate on a rotary shaker for 2 min. This washing procedure was repeated three times. A 200-μl volume of rabbit antitoxin in a 1:200 dilution with 0.85% saline–0.05% Tween 20–1% bovine serum albumin was added to each well and allowed to react with the adsorbed toxin for 2 h at 37°C. The wells were then washed five times as described above. A 200-μl volume of a 1:800 dilution of goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Kirkegaard and Perry Labora-

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tories, Inc.) in PBS–0.005% Tween 20 was added to each well and allowed to react with the rabbit antitoxin for 2 h at 37°C. Finally, after the wells were washed five times as described above, 200 μl of warm substrate (0.1% *p*-nitrophenol phosphate–10% diethanolamine–0.01% $MgCl_2$, pH 9.6) was added to each well, and the reaction was allowed to progress at 37°C for 30 min. The reaction was stopped by adding 50 μl of 2 M NaOH. Release of *p*-nitrophenol was measured spectrophotometrically at 405 nm in an ELISA reader (Bio-Tek Instruments, Inc.). Each indirect ELISA experiment was performed in triplicate with two wells per sample. Negative controls (optical density at 405 nm [OD_{405}] below 0.050) included (i) coating the wells with bovine serum albumin (0.1 to 1 μg per well) or *C. perfringens* α-toxin (Sigma) (0.1 to 1 μg per well) in place of enterotoxin and (ii) the addition of normal rabbit serum (Cappel Laboratories) in place of rabbit antiserum.

(ii) Four-layer sandwich ELISA procedure. Each well of an Immulon II enzyme immunoassay plate was coated with 200 μl of goat antitoxin in a 1:100 dilution in carbonate buffer (0.015 M Na_2CO_3–0.035 M $NaHCO_3$, pH 9.6), and the plates were incubated overnight at 4°C in a humid chamber. After this incubation, the plates were washed once (as described above for the indirect ELISA), and excess binding sites on the microtiter plate were blocked at 37°C for 30 min with 100 μl of 3% bovine serum albumin–1% normal goat serum (Cappel Laboratories) diluted in PBS per well. The blocking solution was removed from each well, and the wells were washed twice as described above. Samples (100 μl per well) containing enterotoxin diluted in 0.05% Tween 20 in PBS were added to each well, and the plates were incubated at 37°C for 2 h. Each well was washed once before a repetition of the blocking procedure (as described above) for 30 min at 37°C. After the blocking procedure, the plates were washed twice as described above, and 200 μl of rabbit antitoxin (diluted as for the indirect ELISA) were added to each well. The antitoxin was allowed to react with the bound enterotoxin in the wells for 2 h at 37°C. Each well was then washed three times as described above, followed by the addition of 200 μl of conjugate (diluted as for the indirect ELISA assay) per well. Conjugate was allowed to react with antitoxin for 2 h at 37°C. Finally, after the wells were washed three times as described above, 200 μl of warm substrate (prepared as described above for the indirect ELISA) were added to each well, and the reaction was allowed to progress at 37°C for 30 min. The reaction was terminated by adding 50 μl of 2 M NaOH. Results were read spectrophotometrically at 405 nm. Each sandwich ELISA experiment was performed in triplicate with two wells per sample. Negative controls (OD_{405} range below 0.050) included coating the wells with normal goat serum (Cappel Laboratories), the addition of bovine serum albumin (0.1 to 1 μg per well) or *C. perfringens* α-toxin (0.1 to 1 μg per well) in place of enterotoxin, and the substitution of normal rabbit serum for rabbit antitoxin.

Detection of enterotoxin in fecal specimens. To demonstrate that the sandwich ELISA could detect enterotoxin in fecal material, we added purified enterotoxin to a fecal specimen prepared by a slight modification of the method of Lyerly et al. (10). Enterotoxin (0.5 to 15 μg in 1 ml of PBS–0.05% Tween 20) was added to a fecal specimen (1 g of feces in 9 ml of PBS–0.05% Tween 20) which was negative for enterotoxin when tested by immunodiffusion (19). The fecal specimen was vortexed well and incubated for 20 min at room temperature. The specimen was then centrifuged (15,000 × *g* for 30 min at 4°C), and the supernatant fluid was passed through a 0.22-μm EGWP membrane filter (Millipore Corp.).

Each fecal ELISA experiment was performed in triplicate with three wells per sample.

Production of enterotoxin by *C. perfringens* strains. Six strains of *C. perfringens* type A were examined for enterotoxin production to demonstrate that the sandwich ELISA differentiated enterotoxin-positive from enterotoxin-negative strains and detected enterotoxin in bacterial culture supernatants. Three enterotoxin-positive strains, NCTC 8239 (Hobbs serotype 3), NCTC 8798 (Hobbs serotype 9), and NCTC 10239 (Hobbs serotype 12), were employed. Enterotoxin-negative strains used included FD-1, 8-1, and ATCC 3624. Details of isolation and characterization of the strains are reported elsewhere (3).

Cultures were grown by a slight modification of the method of Stark and Duncan (20). An actively growing culture of each strain was obtained by transfer from a cooked-meat stock culture to 6 ml of fluid thioglycolate medium. The inoculated fluid thioglycolate medium was heat shocked for 20 min at 70°C, and each culture was subsequently incubated overnight at 37°C. Each fluid thioglycolate medium culture was then transferred to 100 ml of Duncan-Strong sporulation medium (2) and incubated for 14 h at 37°C to obtain release of enterotoxin into the culture supernatant (5). Each Duncan-Strong culture was then rapidly cooled in an ice bath and centrifuged at 10,000 × *g* for 20 min at 4°C. The pellet was discarded and the supernatant was recentrifuged as described above. Each culture supernatant was then filtered through a 0.45-μm membrane filter and diluted 1:2 with PBS containing 0.1% Tween 20. The presence of enterotoxin in culture supernatant fluids was then examined by the sandwich ELISA procedure. Each supernatant ELISA experiment was performed in triplicate with two wells per sample.

RESULTS

Figures 1 and 2 demonstrate that purified *C. perfringens* type A enterotoxin was rapidly and specifically detected by either the indirect (Fig. 1) or sandwich (Fig. 2) ELISA procedures. The indirect ELISA was capable of detecting enterotoxin quantities of as small as 2.5 ng (25 ng/ml), whereas the more sensitive sandwich ELISA procedure

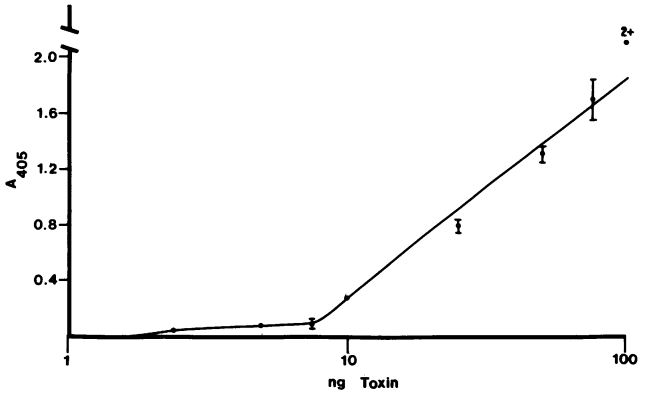

FIG. 1. Sensitivity of the indirect ELISA for the detection of purified *C. perfringens* enterotoxin. The OD at 405 nm plotted for each enterotoxin concentration (as nanograms per well) was determined by subtraction of the mean OD_{405} (less than 0.02) of negative control samples ($n = 6$) from that of test samples ($n = 6$) containing enterotoxin. The bar represents the standard error of the mean for each enterotoxin concentration. (Points without bars had standard errors too small to depict.) A_{405}, Absorbancy at 405 nm.

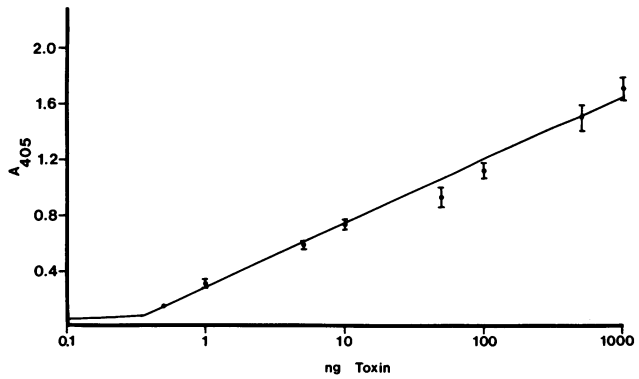

FIG. 2. Sensitivity of the sandwich ELISA for the detection of purified *C. perfringens* enterotoxin. The OD at 405 nm plotted for each enterotoxin concentration (as nanograms per well) was determined by subtraction of the mean OD_{405} (less than 0.02) of negative control samples ($n = 6$) from that of test samples ($n = 6$) containing enterotoxin. The bar represents the standard error of the mean for each enterotoxin concentration. (Points without bars had standard errors too small to depict.) A_{450}, Absorbancy at 405 nm.

specifically detected as little as 0.1 ng (1 ng/ml) of enterotoxin.

To determine the specificity of both the indirect and sandwich ELISA procedures, rigorous controls were employed. Neither bovine serum albumin nor *C. perfringens* α-toxin yielded positive results (positive results were considered to be a blank-corrected OD at 405 nm of >0.050) for either the indirect or the sandwich ELISA procedures, indicating that the goat and rabbit antisera used in these studies possessed high specificities. It is important to note that this assay discriminated between protein toxins (α-toxin versus enterotoxin) produced by *C. perfringens*. Negative ELISA results were obtained when normal goat or rabbit serum was appropriately substituted for immune serum in either the indirect or sandwich ELISA procedure.

Table 1 indicates that the sandwich ELISA procedure specifically detected at least 0.5 μg of enterotoxin per g of feces (50 ng/ml) in prepared fecal samples.

Table 2 demonstrates that *C. perfringens* strains previously classified as enterotoxin-positive by ileal loop assay and immunodiffusion (3) were clearly distinguishable from enterotoxin-negative strains by our sandwich ELISA procedure.

TABLE 1. Detection of *C. perfringens* enterotoxin in human fecal samples by sandwich ELISA

Enterotoxin (μg/g of prepared fecal specimen)[a]	ELISA (OD_{405})[b]
15[c]	0.400 ± 0.034
10	0.338 ± 0.020
5	0.239 ± 0.018
1	0.166 ± 0.019
0.5	0.093 ± 0.014
None	0.015 ± 0.010

[a] Purified enterotoxin was added to fecal specimens prepared as described in the text.

[b] ELISA values are expressed as OD_{405}s ± standard errors of the means. ODs were determined by subtraction of OD_{405}s (OD_{405} <0.02) in negative controls, which received neither enterotoxin nor fecal specimen, from the mean OD_{405} of six test samples. OD_{405}, OD at 405 nm.

[c] This value corresponds to 1.5 μg of enterotoxin per ml.

TABLE 2. Sandwich ELISA values for supernatants from enterotoxin-positive and enterotoxin-negative *C. perfringens* strains

Strain	Dilution[a]	ELISA (OD_{405})[b]
Enterotoxin-positive[c]		
NCTC 8239	Full strength	0.851 ± 0.030
	1:10	0.580 ± 0.047
NCTC 8798	Full strength	0.627 ± 0.047
	1:10	0.429 ± 0.041
NCTC 10239	Full strength	0.892 ± 0.048
	1:10	0.631 ± 0.045
Enterotoxin-negative[c]		
FD-1	Full strength	0.075 ± 0.016
	1:10	0.015 ± 0.012
8-1	Full strength	0.040 ± 0.008
	1:10	0 ± 0
ATCC 3624	Full strength	0.082 ± 0.009
	1:10	0.017 ± 0.005
Duncan-Strong medium	Full strength	0.002 ± 0.002
	1:10	0.004 ± 0.002

[a] Dilutions of culture supernatants were made as described in the text.

[b] ELISA values are expressed as OD_{405}s ± standard errors of the means for 100 μl of culture supernatant or diluted supernatant. OD_{405}s were determined by subtraction of OD_{405}s (OD_{405} <0.02) in negative controls, which received neither enterotoxin nor Duncan-Strong medium, from the mean OD_{405} of six test samples. OD_{405}, OD at 405 nm.

[c] Enterotoxin-positive and enterotoxin-negative strains were classified previously (3).

DISCUSSION

For the period from 1976 to 1980, the Centers for Disease Control reported that 7.5% of the outbreaks (involving 15.8% of the affected persons) of food-borne disease in the United States were due to *C. perfringens* food poisoning (16). It is often difficult to distinguish *C. perfringens* food poisoning without laboratory evidence. Bacteriological examinations are usually employed to diagnose this food poisoning. Since up to 95% of normal adults have *C. perfringens* in their fecal flora (1), isolation of the organism without epidemiological or other support is not sufficient for diagnosis.

Currently, it is desirable to develop a more rapid and convenient method for diagnosis of *C. perfringens* food poisoning. Several methods for the specific detection of *C. perfringens* enterotoxin have been reported (13, 14, 21), but none has yet been extensively used for the investigation of food-borne disease outbreaks. This is at least partially due to the inconvenience of these assays for large-scale application. Additionally, some assays are unsatisfactory for investigations of food-borne disease, owing to interference by fecal material (13, 17).

ELISA procedures are rapid, specific, and sensitive serological assays which have been used for the detection of several bacterial toxins (8–10). During the preparation of our report, Olsvik et al. described an indirect ELISA for the detection of *C. perfringens* enterotoxin (15). However, their ELISA requires a minimum of 4 days for completion. Our indirect and double-sandwich ELISA procedures can be

completed within 24 h. Furthermore, investigations in our laboratory (data not shown) indicate that overnight precoating of ELISA plates with enterotoxin (for the indirect ELISA) or goat antitoxin (for the sandwich ELISA), followed by storage at −70°C for at least 1 week, does not affect ELISA results. Storage of precoated plates for several weeks at −70°C has been successfully reported for an ELISA for *C. botulinum* type G toxin (9). When precoated plates stored at −70°C are used with our enterotoxin ELISA procedures, assay times are reduced to less than 8 h for the sandwich ELISA and less than 6 h for the indirect ELISA, resulting in substantial improvement of the convenience of the enterotoxin ELISA procedures for laboratory usage.

In our current study, the indirect ELISA could easily detect 2.5 ng (25 ng/ml) of purified enterotoxin, whereas the rapid sandwich ELISA procedure was sensitive to 100 pg (1 ng/ml) of purified enterotoxin. By comparison, the highly sensitive sandwich ELISA procedure of Olsvik et al. (15) was approximately 10-fold more sensitive (0.1 ng/ml) for the detection of purified enterotoxin. Apparently there is some trade-off in assay sensitivity when our rapid procedure is used.

Our sandwich ELISA procedure easily detected 0.5 μg of enterotoxin per g of feces (50 ng/ml) in prepared fecal extracts. The presence of fecal material apparently inhibits the sensitivity of our sandwich ELISA (Fig. 2; Table 1). However, since the detectable amount of enterotoxin in fecal samples from *C. perfringens* food poisoning cases is reported to be approximately 0.5 to 16 μg/g of feces (by counterimmunoelectrophoresis, electroimmunodiffusion, and reversed passive hemagglutination) (17, 18), the sandwich ELISA described in our report should be sufficiently sensitive (minimum sensitivity, 0.5 μg/g of feces) to be useful for the rapid detection of enterotoxin in feces.

Table 2 indicates that the sandwich ELISA specifically differentiated enterotoxin-positive from enterotoxin-negative *C. perfringens* strains and that Duncan-Strong medium does not interfere with the sandwich ELISA, demonstrating that the indirect ELISA is useful for the examination of supernatants of *C. perfringens* cultures. Very weakly positive ELISA results for supernatants from some enterotoxin-negative strains could indicate very low levels of enterotoxin production, serological cross-reactivity with small quantities of contaminating spore coat protein (4), or possibly the presence of trace contaminants in the purified enterotoxin.

In summary, we report indirect and sandwich ELISA procedures for the rapid detection of *C. perfringens* enterotoxin. The present study confirms the earlier ELISA work of Olsvik et al. (15) in regard to the suitability of ELISAs for the detection of enterotoxin in buffers, feces, or culture supernatants. Furthermore, our procedures offer a considerable savings in assay time over the methods of Olsvik et al. (15). Although the method of Olsvik et al. is about 10-fold more sensitive, our rapid sandwich ELISA procedure retains sufficient sensitivity for nearly all anticipated uses, including rapid enterotoxin detection from fecal material or culture supernatants.

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