Assay Methods for *Clostridium perfringens* Type A Enterotoxin

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Enterotoxin produced by a sporulating culture of *Clostridium perfringens* type A NCTC 8798 was purified to a level of 3,500 mouse mean lethal doses per mg of nitrogen. High-titer sera were obtained from rabbits injected with enterotoxin and used to compare the sensitivity of serological tests and bioassays for *C. perfringens* enterotoxin. Reversed passive hemaggulination was by far the most sensitive test, followed by microslide diffusion, single gel diffusion and electrophromunodiffusion, guinea pig skin test, mouse test, and rabbit ileal loop test.

Sporulating cultures of food poisoning *Clostridium perfringens* type A contain a factor that produces enteropathogenic reactions in ligated intestinal loops of lambs and rabbits, food poisoning symptoms in monkeys and humans, erythema in guinea pig and rabbit skin, and is lethal to mice (7, 8, 12, 13, 19–22). Purified preparations of the toxic factor have been produced and the toxin (enterotoxin) has been identified as a heat-sensitive protein of molecular weight of about 35,000 (16, 21, 22). Detection of enterotoxin is currently accomplished by a number of biological and serological tests of different sensitivity. Based on the erythema activity of enterotoxin (12, 16), a guinea pig or rabbit intradermal skin test has been developed which allows detection of at least 2.5 μg of enterotoxin per ml. A minimum amount of about 0.125 μg is needed to cause erythema reaction in the skin. The test is rapid, relatively inexpensive, reproducible, and accurate (12) but not specific. With rare exceptions mice injected intravenously (i.v.) with enterotoxin either died within 20 to 30 min or they were not killed at all. The minimal lethal dose for mice by the i.v. route is about 3 μg of purified enterotoxin (16, 21). Ligated intestinal loops of rabbits and lambs have been used not only for the detection of enterotoxin in culture media but also for the identification of enterotoxigenic strains of *C. perfringens* type A (7, 10, 14, 16, 20). Enteropathogenic reaction has been produced in ligated intestinal loops of rabbits (24-h standard procedure) by a minimum of 125 μg of enterotoxin (12) and 28 to 40 μg (21). A more rapid (90 min) ligated intestinal loop test in rabbits required at least 6.25 μg of enterotoxin for enteropathogenic reaction (15). Bartlett et al. (2) reported the use of dogs for the detection of enterotoxin in culture media. From their data it can be estimated that more than 100 μg of enterotoxin was required to cause enteropathogenic response. The biological tests have been valuable in the research that led to the discovery of enterotoxin in *C. perfringens* cultures. These tests are, however, not specific.

The research for serological methods for the detection of *C. perfringens* type A enterotoxin has been facilitated by the recent availability of anti-enterotoxin sera made specific by appropriate absorptions (9, 13, 15, 19–21). The Ouchterlony double-immunodiffusion method performed on standard microscope slides allows identification of enterotoxin produced by *C. perfringens* type A (10, 18, 20). This method is not as sensitive as the recently reported electroimmunodiffusion or single gel diffusion tube methods (5) which can detect 1 and 3 μg of enterotoxin per ml, respectively.

This paper presents some of our experiences in adapting known serological tests for fast, specific, and sensitive detection of *C. perfringens* type A enterotoxin. In addition, the various methods of mass screening for enterotoxin-producing *C. perfringens* were compared.

**MATERIALS AND METHODS**

*Cultures.* *C. perfringens* type A strain NCTC 8798 was obtained from C. L. Duncan, Food Research...
Institute, University of Wisconsin, Madison, Wis., and used for the production of enterotoxin.

**Production of sporing cells.** Five hundred milliliters of semi-solid (0.3% agar) (DS) sporing medium (6) with no phosphate added was inoculated with 20 ml of a 24-h (35 C) fluid thioglycolate culture. The DS medium was incubated in a 6-liter flask for 12 to 16 h at 35 C. Prewarmed (37 C) liquid DS medium (5.5 liters) with phosphate supplement, was then added and the incubation was continued until more than 50% of the cells had produced refractile spores. This usually took 5 to 7 h. The sporing culture was cooled in a walk-in refrigerator at 2 C; the cells were harvested by centrifugation in the cold and washed once with cold saline. A total of 24 liters of culture was produced and the washed cell crop was frozen in 800 ml of saline at -18 C.

**Purification of enterotoxin.** Purification was accomplished by a modification of the procedure by Stark and Duncan (20) and will be reported in detail elsewhere (Sakaguchi et al., unpublished data). In brief, the purification of enterotoxin was performed by using the following steps: (i) sonic treatment of defrosted cells for 30 min and separation of the enterotoxin containing fluid by centrifugation; (ii) precipitation of enterotoxin in the supernatant fluid with 40% saturated (NH4)2SO4, pH 7.0, overnight in the cold; (iii) up-flow filtration of redissolved toxin on Sephadex G-200, with sodium phosphate buffer (0.02 M), pH 6.7, at room temperature; and (iv) precipitation by 75% saturated (NH4)2SO4, pH 7.0, and repeated filtration on Sephadex G-200. Toxic fractions as determined by mouse i.v. test were pooled, concentrated with Carboxaw 20,000 (Union Carbide Corp.) at room temperature, distributed in small screw-cap tubes, and frozen. The protein concentration determined by Lowry's method (17) was 0.29 mg per ml in the final toxin preparation.

**Production of antisera.** Three young New Zealand white rabbits (4 pounds; about 1,816 g) were inoculated with the purified enterotoxin. At 3-day intervals each rabbit received in order, 2.9, 12, 29, and 29 μg of enterotoxin dissolved in 1 ml of saline and mixed on a Vortex mixer with 1 ml of complete Freund adjuvant. Half of the antigenic preparation was injected intramuscularly, the other half subcutaneously. One month after the last injection the rabbits were challenged with 145 μg of enterotoxin in saline injected subcutaneously. Beginning 1 week after the last injection, three 45-ml samples of blood were drawn from the heart of each rabbit over a 15-day period. The sera of the three rabbits were mixed, labeled as batch no. 1, and used in the present study. Additional challenges with 145 μg of enterotoxin were later performed at 2-month intervals. Clostridium diagnostic sera, C. perfringens types A, B, C, D, and E were obtained from Wellcome Research Laboratories.

**Specificity of antiserum, immunodiffusion tests.** The specificity of the prepared antiserum was tested by the microslide double-gel diffusion method (4). The gel was prepared according to Casman et al. (4). The microslide assembly was the one reported by Untermann (24).

The single gel diffusion technique was applied for quantitative estimation of C. perfringens enterotoxin in sporing media and rabbit ileal loop contents.

Single-gel diffusion tubes (3.8 mm internal diameter and 100 mm length) containing 0.3 ml of an antiserum-agar dilution were prepared as described before (11, 25), using Noble agar (Difco). The concentration of agar was 0.3% in standard buffer (phosphate-buffered saline, pH 7.2, 0.02 M with 1:10,000 Merthiolate). The concentration of antiserum in the agar varied from 1:20 to 1:200. A standard enterotoxin reference curve for each antiserum concentration was constructed by adding to duplicate-gel diffusion tubes 0.3 ml of the standard buffer containing amounts of purified enterotoxin from 0.5 to 100 μg per ml. The tubes were sealed with plasticine, incubated at 30 C, and checked at 12, 24, and 48 h. The migration distance of the toxin-antitoxin precipitin band was plotted against the log of the toxin concentration. The highest dilution of antiserum that resulted in uniformly dense bands was selected for preparing gel-diffusion tubes for routine toxin determination. The tubes were stored in the refrigerator with no change in activity of antiserum for over a year.

**Hemaggulatination.** Enterotoxin or specific IgG antibody sensitization of formalinized sheep erythrocytes was based on the use of bis-diazo-benzidin (BDB) as described by Butler (3) with modification in the concentrations of BDB, enterotoxin, and antibody (Uemura and Riemann, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 22).

**Determination of toxin potency.** (i) **Mouse LD₅₀.** Enterotoxin preparations were diluted in 0.02 M phosphate buffer, pH 6.7, to concentrations, in a geometric progression, from 5 to 12 μg per ml (17). Then 0.25 ml of each dilution was injected i.v. into six White Swiss Webster male mice, weighing 17 to 20 g, and the number of deaths was recorded. The mean lethal dose (LD₅₀) was calculated from the proportion of deaths according to the method of moving averages (23).

(ii) **Rabbit ligated intestinal loop.** The rabbit intestinal loop technique was performed as previously described (10). The young New Zealand white rabbits used weighed about 2 to 3 pounds (about 1,128 g) and were fasted for 72 h before the operation and kept for 24 h after. Twofold dilutions of purified enterotoxin were made to 3 ml with saline and injected intraluminally, each in 10-cm-length intestinal segments. A 3-ml inoculum has been used routinely for enterotoxicity studies of various C. perfringens strains as well as neutralization studies (Torres et al., 1973, Mem. 7th Pan. Vet. Congress).

(iii) **Erythema activity.** Erythema activity was determined in guinea pig skin as reported by Stark and Duncan (20). To enhance the reading of the skin reaction, 1 ml of 0.5% Evans blue was injected into the heart 10 min after the skin was injected. The diameter of reaction zone was measured 80 min later.

**Neutralization of biological activity of enterotoxin.** Twofold dilutions of the antitenterotoxin serum were mixed 1:1 with various concentrations of purified enterotoxin in saline. The mixtures were incubated 60 min at room temperature, then cen-
trifuged, and the supernatant fluid was injected intravenously into mice (0.25 ml), into rabbit ligated intestinal loop (3 ml), and into guinea pig skin (0.05 ml). *C. perfringens* diagnostic antitoxins (types A, B, C, D, and E) were used as controls for the specificity of the enterotoxin-antienterotoxin reaction.

**RESULTS**

The yield of toxin preparation from 24 liters of culture was 13 mg of protein. The LD₉₀ for mice was 1.8 µg of protein. This corresponds to 3,527 LD₉₀ per mg of N in the preparation and is somewhat higher than the minimal lethal dose of 2,100 and 2,232 per mg of N quoted by Hauschild et al. (16) and Stark and Duncan (21), respectively, but it was observed that the sensitivity of mice is quite dependent on their size. A positive rabbit intestinal loop response was obtained with a minimum of 29 µg of enterotoxin. Easily recognized reactions (16) of 5-, 6.5-, and 9.3-mm diameter were caused by injection of 0.06, 0.125, and 0.25 µg of enterotoxin intradermally in the guinea pig skin. One milliliter of the produced antiserum neutralized the toxicity of 145, 500, and 603 µg of enterotoxin to rabbit intestinal loop, guinea pig skin, and mice, respectively. Diagnostic *C. perfringens* antitoxins had no detectable neutralizing power against enterotoxin.

A single line of precipitation was obtained with as much as 290 µg of enterotoxin per ml tested by the microslide procedure against 1:25 and higher dilutions of the antiserum, whereas at least four lines of precipitation (two major, two minor) appeared in the microslide when undiluted serum was tested against 290 µg of enterotoxin per ml. There was only a single precipitation line with undiluted serum when commercial Ouchterlony immunodiffusion plates (Hyland Laboratories, Los Angeles, Calif., patterns C and D) as well as standard microscope slide techniques (agar same as the microslide agar) were used.

The single-gel diffusion test permitted detection of 0.50 to at least 290 µg of enterotoxin per ml with up to 1:200 diluted antiserum. However, for routine work a 1:75 dilution of antiserum in the gel was chosen because of the easier detection of precipitation bands. As expected (25), the width of the bands was influenced by a number of variables. The width of the bands increased with increasing concentrations of enterotoxin, ionic strength, temperature, and time of incubation. The pH effect (pH 5–8) was not significant. A straight-line regression was obtained when enterotoxin concentration from 5 to 200 µg per ml in standard buffer was plotted on semilog paper as the ordinate and the band migration in 24 h at 30 C as the abscissa. Below the lower limit the line curved sharply downward. The regression line allowed quantitative estimation of enterotoxin present in standard buffer under constant conditions. Large assay errors occurred if the toxin was present in diluents that contained solutes of different composition and concentration (Fig. 1). Therefore, to obtain correct assay values the same diluent must be used for preparing standard curves and for diluting samples of culture supernatant fluids. If dilution of the toxic preparation is undesirable, dialysis should be carried out before assay against the buffer for which the standard curve was obtained. Dialysis is time consuming, however, and an alternative is to use toxin dissolved in sporulation medium or solution of similar effect for construction of the standard curve.

As shown in Fig. 1, the DS sporulation medium used for the production of enterotoxin has a similar effect on the width of the precipitation band as the standard buffer plus 1 M NaCl. A regression line based on the latter can be used for assaying enterotoxin produced in this sporulation medium.

Erythrocytes sensitized with specific antibodies or enterotoxin were used successfully for the detection of enterotoxin and for the determination of antienterotoxin titers in human and animal sera by hemagglutination test. Sera titers of immunized rabbits ranged from 1:620,000 to 1:2,560,000. Erythrocytes sensitized with specific antibody permitted the detection of as little as 0.0005 to 0.001 µg of enterotoxin per ml by the reverse passive hemagglutination (RPH) method (Uemura and

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**Fig. 1.** Effect of buffer molarity on the migration of enterotoxin-antienterotoxin precipitin bands. Symbols: O, PBS; ●, PBS plus 0.1 M NaCl; □, PBS plus 0.4 M NaCl; Δ, PBS plus 1 M NaCl.
Riemann, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 22.

A comparison of the sensitivity and the antigen volumes required in the tests evaluated for the detection of enterotoxin as well as two methods reported by others are presented in Table 1.

**DISCUSSION**

The immunization schedule and doses used indicate that only small amounts of enterotoxin are needed to make the rabbits respond with the production of high-titer antisera. Similar schedules and doses have been found satisfactory for the production of high-titer antisera against staphylococcal enterotoxins A, B, and C. Administration of large doses practiced by some investigators might result in the production of low-titer antisera due to the immunological paralysis of the rabbits.

The comparison of the tests available for the detection of enterotoxin indicates that by far the most sensitive test is the RPH. This test is currently used for mass screening of enterotoxin producing *C. perfringens* in foods, human stools, animal intestinal content, environment, and food poisoning outbreaks. The sensitivity of RPH, microslide gel, and single-gel immunodiffusion tests for detecting *C. perfringens* enterotoxin resembles the sensitivity of these tests for detecting staphylococcal enterotoxins (1). Using dilutions of antisera from 1:75 to 1:200 for the single gel immunodiffusion tubes, as little as 0.9 µg of enterotoxin per ml could be detected. Smaller amounts of enterotoxin can be detected if the toxin is present in buffers with molarity higher than the standard. Standard buffer plus 0.5 M, 1 M, and 2 M NaCl was found equally satisfactory in permitting detection of 0.5 µg of enterotoxin per ml. This sensitivity per milliliter is similar to electroimmunodiffusion (9), but the latter requires 30 times less antigen and 6 times more antisera gel. The single-gel diffusion is an excellent and simple quantitative test for *C. perfringens* enterotoxin. Depending on the degree of sporulation in DS medium, *C. perfringens* type A, strain NCTC 8239, NCTC 8798, and NCTC 10239 produced up to 1, 100, and 14 µg of enterotoxin per ml, respectively, after 24 to 36 h of incubation as determined by single-gel diffusion. No doubt, for a trained person, the microslide immunodiffusion test with the small volume of reagents needed, its sensitivity, and the possibility of comparing known with unknown antigens should be considered a method of choice, in most cases, for qualitative analysis. The test has been used successfully for quantitative detection of staphylococcal enterotoxins (4). However, considering sensitivity and speed, the RPH is the superior technique. The serological tests are the most specific tests for *C. perfringens* type A enterotoxin. Yet these tests cannot detect antigenically different enterotoxins (which might exist) unless specific antisera for these enterotoxins are available. The biological tests based on erythema activity, mouse lethality, and rabbit ileal loop reaction are, therefore, important tools for detection of new serological types of enterotoxins.

It is interesting to notice that our antisera has a high titer in the microslide and single-gel diffusion tests as compared to the one produced by Duncan and Somers (5). On the other hand, its neutralizing power of 500 µg of enterotoxin per ml of serum is much weaker than the approximately 5,300 µg of enterotoxin per ml of serum reported by these authors. These discrepancies might be due to different reagents used with respect to type, concentration, and size of gel, or different concentration, and size of gel, or different concentration of immunoglobulin M and G antibodies in the antiserum.

**TABLE 1.** Comparison of serological and biological methods used for the detection and quantitation of *C. perfringens* type A enterotoxin

| Method                        | Minimal enterotoxin detection | Extra-polated minimal concn. of enterotoxin (µg/ml) |
|-------------------------------|-------------------------------|--------------------------------------|
|                               | Enterotoxin (µg) | Dose volume (ml) |                                  |
| Reverse passive hemagglutination | 0.00005          | 0.05               | 0.001                             |
| Erythemal activity            | 0.06-0.125        | 0.05               | 1.25-2.50                         |
| Microslide diffusion          | 0.013             | 0.025              | 0.50                              |
| Single gel diffusion          | 0.30              | 0.30               | 0.9*                              |
| Mouse lethality (LD₅₀)         | 1.8               | 0.25               | 6.8                               |
| Rabbit ileal loop (standard)   | 29                | 3.0                |                                   |
| Rabbit ileal loop (90 min)     | 6.25              | 2.5                |                                   |
| Electroimmuno diffusion (9)    | 0.01              | 0.01               | 1.0                               |

* Skin reaction of at least 0.5 cm in diameter read 90 min after injection.
* Band width of 1 mm from miniscus, incubation for 24 h at 30 C, antisera gel 1:100.

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