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Vero Cell Assay for Rapid Detection of *Clostridium perfringens* Enterotoxin

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A rapid assay which measured the biological activity of *Clostridium perfringens* enterotoxin was developed. The method involved the rapid killing of Vero cells by enterotoxin produced by *C. perfringens* grown in Duncan and Strong sporulation medium. Serial dilutions of toxin were added to Vero cells either in suspension or grown as monolayers in wells of a 96-well cell tissue culture cluster plate. Vital staining of Vero cells with neutral red, followed by extraction of the dye, allowed toxin levels to be determined either visually or by optical density measurements with a micro-ELISA M580 computer program. The toxin produced was confirmed as different from the Vero toxin of *Escherichia coli* and the alpha and theta toxins of *C. perfringens*.

Clostridium perfringens is the second most common cause of bacterial food poisoning outbreaks worldwide (16). *C. perfringens* is normally found in large numbers in soil, sewage, and the intestinal tracts of humans and animals. When the clinical symptoms of food poisoning are present, high levels of *C. perfringens* organisms in the feces (10^6 bacteria per g of feces) may be indicative of *C. perfringens* food poisoning. It has been shown, however, that geriatric populations tend to have very high fecal counts of *C. perfringens* (7, 17, 20). Under such circumstances, enterotoxin detection may be the only definitive method of diagnosing *C. perfringens* gastroenteritis.

Enterotoxin has traditionally been associated with sporulation in *C. perfringens*, although the relationship is unclear (5, 9, 11). Various media conducive to sporulation have been developed, but there is no single universal medium that will encourage sporulation in all strains (16). Some strains may sporulate well in Duncan and Strong medium (2); others may sporulate better when the starch in Duncan and Strong medium is replaced with raffinose (10) or when Tórtora medium is used instead (18).

Three categories of tests can be used to detect *C. perfringens* enterotoxin: biological tests, serological tests, and tissue culture assays. In this study we have chosen to further examine tissue culture assays. These assays involve the production of cytotoxic effects on various cell lines. The continuously cultured cell line of African green monkey kidney cells (Vero cells) is very sensitive to *C. perfringens* enterotoxin. McClane and McDonel (13) first described the effects of enterotoxin on Vero cells, which included morphological changes, detachment from glass surfaces, decreased viability and plating efficiency, and altered macromolecular synthesis. They observed that enterotoxin had a very rapid action on Vero cells, bringing about membrane blebs and a net influx of ions and water into the cells. McDonel and McClane (15) subsequently developed a sensitive enterotoxin assay in which nanogram amounts of enterotoxin could be detected by microscopically observing a decrease in the plating efficiency of toxin-treated Vero cells. Other methods of recording Vero cell sensitivity to *C. perfringens* enterotoxin have been described (3, 19), but all require microscopic observation of the cells. In this paper we present further experience with a Vero cell system and describe a rapid and easily interpreted assay for *C. perfringens* enterotoxin.

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MATERIALS AND METHODS

Strains of *C. perfringens*. Strains of *C. perfringens* were grown in cooked-meat medium (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C. These cultures were then stored at room temperature and subcultured to fresh cooked-meat medium when required. Toxin-producing strains were isolated during a hospital outbreak of *C. perfringens* diarrhea and were originally obtained from M. F. Stringer, Central Public Health Laboratory, London, England. One of these (strain B35 in our collection) was used as a known toxin-producing strain. Other strains of unknown toxin-producing capability were part of our collection.

Media. Brain heart infusion broth (BHI; Difco) was used to grow *C. perfringens* from overnight cooked-meat cultures. Duncan and Strong sporulation medium was used to induce sporulation (2). Growth medium for the Vero cells consisted of minimum essential medium (MEM; Gibco/BRL, Life Technologies, Inc., Burlington, Ontario, Canada) containing streptomycin and penicillin and supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, Mississauga, Ontario, Canada). Maintenance medium used in the enterotoxin assay consisted of MEM containing 1% FBS.

Cell culture. Vero cells used in this study were originally obtained from K. McCarthy, University of Liverpool, Liverpool, England. These cells were routinely cultured at 37°C in a humidified incubator containing 5% CO_2.

Production of *C. perfringens* enterotoxin. A 10-fold dilution of a fresh overnight cooked-meat culture was made in freshly boiled and cooled BHI broth. After incubation for 3 h at 37°C, a 10-fold dilution of this culture was made into freshly boiled and cooled Duncan and Strong broth. The Duncan and Strong culture was incubated for 18 h and then centrifuged at 6,000 × *g* for 10 min, and the supernatant fluid was collected. The enterotoxin could be concentrated from the culture supernatant by ammonium sulfate precipitation (28 g/100 ml of supernatant at 4°C for 24 h). The precipitate was collected by centrifugation, suspended in distilled water (1/40 original volume), and then exhaustively dialyzed against distilled water. Floccular material observed in the dialysate was removed by centrifugation (without loss of toxin titer), and the supernatant fluid was filter sterilized and stored at 4°C.

Vero cell assay for enterotoxin. Twofold dilutions of toxin were made in MEM containing 0.01% neutral red dye and 1% FBS, and 50-μl volumes of these dilutions were added to a monolayer of Vero cells which had been grown for 24 h in a 96-well tissue culture cluster plate (Costar, Cambridge, Mass.). The plate was incubated in a CO_2 incubator for 2 h at 37°C. After incubation, excess dye was removed by washing the cells three times with phosphate-buffered saline, and then 50 μl of phosphate-buffered ethanol (0.1 M NaH_2PO_4–ethanol; 1:1) was added to each well to extract the dye from the viable cells. The color reactions were read visually or by using a micro-ELISA reader that measured the optical density of each well at a transmitting wavelength of 570 nm.

To determine how the concentration of Vero cells might affect toxin titers, we used a range of cell concentrations extending from 3,125 to 400,000 cells per culture plate well to establish monolayers. A series of toxin dilutions was added to such monolayers, and the toxin titers were compared. An alternative method of toxin titration involved the use of a suspension of Vero cells rather than monolayers. Toxin was diluted in MEM containing 10% FBS, and 50 μl of each dilution was added to 100 μl of freshly trypsinized and appropriately diluted Vero cells. The same cell suspensions and toxin dilutions described above for monolayers were used in these experiments. After overnight growth, the fluid was removed from the plates and 50 μl of MEM containing 1% FBS and 0.01% neutral red dye was added to the monlayers that had developed. The plates were incubated and treated for color reactions as previously described.

Neutralization of alpha and theta toxins and enterotoxin. We previously described the assays for alpha toxin (lecithinase C) and theta toxin, in which egg yolk solution and sheep erythrocytes, respectively, were used (12). These toxins were neutralized by mixing 50 μl of culture supernatant with 50 μl of antitoxin (*C. welchii* Type A Clostridial Diagnostic Serum; Wellcome Laboratories, Beckenham, England) and incubating the mixture for 20 min at 37°C before performing the assay. Similarly, *C. perfringens* antienterotoxin antiserum (a gift of C. Duncan) was tested for neutralization of the Vero toxin activity.

RESULTS

Vero toxin assay for *C. perfringens* enterotoxin. The Vero toxin assay provided a very rapid means of detecting *C. perfringens* enterotoxin. The vital dye, neutral red, facilitated determination of the titer, and the micro-ELISA computer reader provided endpoints which were similar to those obtained visually. The titer was expressed as the reciprocal of the toxin dilution that reduced the optical density of the test well to 50% of the control well values. Visual determination of the titer consisted of choosing the well that was intermediate between colorless and intense pink. Usually there was only one such well between a completely positive and a completely negative well. Both methods consistently yielded the same results. Enterotoxin titers could be read as early as 15 min, but plates that were incubated for 60 min were much easier to read; staining was slightly more intense after 2 h. Ammonium sulfate-precipitated toxin had titers up to 30,000 U/ml, and one sample whose titer was determined weekly showed no loss in biological activity over a 6-week period. Protein determinations made on this crude material showed that 25 to 50 ng of toxin-containing protein was detectable.

The number of Vero cells used in cell suspension or to prepare monolayers had a marked effect on the toxin titers (Table 1). An eightfold increase in titer was observed when the number of cells per well was decreased from 400,000 to 25,000. The cell suspension method was twice as sensitive as the monolayer method, and cell numbers below 25,000 per well were not adequate for toxin detection by either method. We have routinely used 50,000 cells per well in our toxin assay.

TABLE 1. Enterotoxin titers obtained with different concentrations of Vero cells in suspension and by the monolayer formation method

No. of cells/well	Toxin titer	
	Cell suspension method[a]	Monolayer method[b]
400,000	2,000	2,000
200,000	4,000	2,000
100,000	8,000	4,000
50,000	16,000	8,000
25,000	32,000	16,000

[a] Cells were treated with toxin for 24 h and then stained.
[b] Cells were treated with toxin for 1 h and stained simultaneously.

Alpha and theta toxins had no effect on Vero cells (Table 2). Neither enterotoxin nor spores were produced in BHI broth, whereas both alpha and theta toxins were produced. The Type A antiserum neutralized both alpha and theta toxins in BHI broth and in Duncan and Strong medium. This antiserum had no neutralizing activity against the Vero toxin activity of *C. perfringens*, which was produced in association with the appearance of spores in Duncan and Strong medium. Conversely, the *C. perfringens* antienterotoxin antiserum did not neutralize the alpha or theta toxins, but only the Vero toxin activity. An antiserum against *E. coli* Vero toxin (kindly provided by M. Karmali, University of Toronto, Toronto, Ontario, Canada) was tested and had no neutralizing activity against the enterotoxin of *C. perfringens* (data not shown), nor did uninoculated control media have any inhibitory activity on Vero cells. MRC-5 cells (one of the cell lines used to detect *C. difficile* cytotoxin) were not affected when challenged with the *C. perfringens* enterotoxin. When unconcentrated 10-ml volumes of Duncan and Strong culture supernatants were obtained from several strains of *C. perfringens* grown under the same conditions,

TABLE 2. Alpha, theta, and enterotoxin titers produced by *C. perfringens* B35 in BHI broth and Duncan and Strong medium

Material tested[a]	Toxin titer[b]		
	Alpha toxin	Theta toxin	Enterotoxin
BHI broth control	0	0	0
BHI culture supernatant	128	512	0
BHI culture supernatant + Type A antiserum[c]	0	0	0
BHI culture supernatant + antienterotoxin antiserum	128	512	0
DS broth control	0	0	0
DS culture supernatant	32	256	256
DS culture supernatant + Type A antiserum	0	0	256
DS culture supernatant + antienterotoxin antiserum	32	256	0

[a] DS, Duncan and Strong. Growth in BHI broth was for 3 h; growth in Duncan and Strong medium was for 18 h.
[b] Titers are expressed as the reciprocal of the toxin dilution.
[c] *C. welchii* Type A Clostridial Diagnostic Serum (Wellcome).

Vero toxin titers ranged from 0 to 512 (data not shown), which reflected the ability of the strains to produce toxin in this medium. Strains capable of producing a high titer did so reproducibly.

DISCUSSION

Cytotoxic activity against Vero cells has been reported to be a sensitive means of detecting *C. perfringens* enterotoxin. The rapidity and mode of action of the toxin have been described previously (4, 6, 8, 13, 14). Although workers in some laboratories have indicated an application for Vero cells in the detection of the enterotoxin, a convenient and rapid technique for detecting the toxin required further attention. Our objective was to acquire experience with the Vero toxin assay and to describe a method which was simple to perform and interpret. We have shown that when a vital dye is incorporated at the time of addition of toxin to Vero cells, toxin titers can be determined in as little as 15 min, although a 1-h incubation period gives better color intensity. If the Vero cell suspension method rather than the monolayer method is used for toxin titer determination, the titer obtained is twofold higher. Although the monolayer method is slightly less sensitive, we found it to be more convenient, since plates could be prepared in advance of the toxin testing. Toxin titers could also be determined microscopically by observing the failure of Vero cell suspensions to attach and spread on the bottom of the cell culture plate after 2 h of incubation or by observing morphological changes in an existing monolayer; however, such observations are more tedious and require experience in reading and interpreting the results. We have not compared the sensitivity of this Vero system with that of other existing methods or worked with purified toxin; however, Berry et al. (1) have compared different assay methods for *C. perfringens* enterotoxin and found that their Vero cell assay system (3) was less sensitive than the enzyme-linked immunosorbent assay and reversed passive latex agglutination test for detecting this toxin in stool specimens.

The fact that *C. perfringens* enterotoxin reacts quickly on Vero cells differentiates it from other Vero-active toxins such as those of *C. difficile*, *Escherichia coli*, and *Shigella* spp. The last two toxins require incubation periods of at least 24 to 48 h before titers are read, and *C. difficile* titers are usually read after an overnight incubation. We found that MRC-5 cells, used in our laboratories for the detection of *C. difficile* cytotoxin, were resistant to the *C. perfringens* enterotoxin. This supports the findings of Giugliano et al. (4) that MRC-5 cells are weakly sensitive to *C. perfringens* enterotoxin. Failure to neutralize the *C. perfringens* enterotoxin with an antiserum prepared against an *E. coli* Vero toxin further differentiates these toxins.

For laboratories with tissue culture facilities, the Vero cell cytotoxin assay provides a rapid and convenient test for the enterotoxin of *C. perfringens*.

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LITERATURE CITED

1. **Berry, P. R., J. C. Rodhouse, S. Hughes, B. A. Bartholomew, and R. J. Gilbert.** 1988. Evaluation of ELISA, RPLA, and Vero cell assays for detecting *Clostridium perfringens* in faecal specimens. J. Clin. Pathol. **41**:458–461.
2. **Duncan, C. L., and D. H. Strong.** 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**: 82–89.
3. **Giugliano, L. G., G. F. Mann, and B. S. Drasar.** 1982. Other enterotoxic enteropathies—the use of tissue culture for the detection of clostridial toxins. Eur. J. Chemother. Antibiot. **2**:139–142.
4. **Giugliano, L. G., M. F. Stringer, and B. S. Drasar.** 1983. Detection of *Clostridium perfringens* enterotoxin by tissue culture and double-gel diffusion methods. J. Med. Microbiol. **16**:233–237.
5. **Goldner, S. B., M. Solberg, S. Jones, and L. S. Post.** 1986. Enterotoxin synthesis by nonsporulating cultures of *Clostridium perfringens*. Appl. Environ. Microbiol. **52**:407–412.
6. **Horiguchi, Y., T. Uemura, S. Kozaki, and G. Sakaguchi.** 1985. The relationship between cytotoxic effect and binding to mammalian cultured cells of *Clostridium perfringens* enterotoxin. FEMS Microbiol. Lett. **28**:131–135.
7. **Jackson, S. G., D. A. Yip-Chuck, J. B. Clark, and M. H. Brodsky.** 1986. Diagnostic importance of *Clostridium perfringens* enterotoxin analysis in recurring enteritis among elderly, chronic care psychiatric patients. J. Clin. Microbiol. **23**:748–751.
8. **Jarmund, T., and W. Telle.** 1982. Binding of *Clostridium perfringens* enterotoxin to hepatocytes, small intestinal epithelial cells and Vero cells. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **90**:377–381.
9. **Labbé, R. G.** 1980. Relationship between sporulation and enterotoxin production in *Clostridium perfringens* type A. Food Technol. **34**:88–90.
10. **Labbé, R. G., and D. K. Rey.** 1979. Raffinose increases sporulation and enterotoxin by *Clostridium perfringens* type A. Appl. Environ. Microbiol. **37**:1196–1200.
11. **Loffler, A., and R. G. Labbé.** 1985. Isolation of an inclusion body from sporulating, enterotoxin-positive *Clostridium perfringens*. FEMS Microbiol. Lett. **27**:143–147.
12. **Mahony, D. E.** 1977. Stable L-forms of *Clostridium perfringens*: growth, toxin production, and pathogenicity. Infect. Immun. **15**:19–25.
13. **McClane, B. A., and J. L. McDonel.** 1979. Effects of *Clostridium perfringens* enterotoxin on morphology, viability, and macromolecular synthesis in Vero cells. J. Cell. Physiol. **99**:191–200.
14. **McDonel, J. L., and B. A. McClane.** 1979. Binding versus biological activity of *Clostridium perfringens* enterotoxin in Vero cells. Biochem. Biophys. Res. Commun. **87**:497–504.
15. **McDonel, J. L., and B. A. McClane.** 1981. Highly sensitive assay for *Clostridium perfringens* enterotoxin that uses inhibition of plating efficiency of Vero cells grown in culture. J. Clin. Microbiol. **13**:940–946.
16. **Stringer, M. F.** 1985. *Clostridium perfringens* type A food poisoning, p. 117–143. *In* S. P. Borriello (ed.), Clostridia in gastrointestinal disease. CRC Press, Inc., Boca Raton, Fla.
17. **Stringer, M. F., G. N. Watson, R. J. Gilbert, J. G. Wallace, J. E. Hassall, E. I. Tanner, and P. P. Webber.** 1985. Faecal carriage of *Clostridium perfringens*. J. Hyg. **95**:277–288.
18. **Tórtora, J. C. O.** 1984. Alternative medium for *Clostridium perfringens* sporulation. Appl. Environ. Microbiol. **47**:1172–1174.
19. **Welch, A. R., S. P. Borriello, and F. E. Barclay.** 1985. Simplified procedure for tissue culture in routine detection of cytotoxins. J. Clin. Pathol. **38**:835–837.
20. **Yamagishi, T., T. Serikawa, R. Morita, S. Nakamura, and S. Nishida.** 1976. Persistent high numbers of *Clostridium perfringens* in the intestines of Japanese aged adults. Jpn. J. Microbiol. **20**:397–403.