

A Research Note

Evaluation of a Reversed Passive Latex Agglutination Test Kit for *Clostridium perfringens* Enterotoxin

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(Received for publication November 27, 1985)

ABSTRACT

A reversed passive latex agglutination (RPLA) test kit for *Clostridium perfringens* enterotoxin (CPE) marketed by the Denka-Seiken Co., Tokyo, Japan, was evaluated by using culture supernatant fluids and extracts from feces of food poisoning patients. Nanograms of CPE were detectable with the assay and the reaction was specific, as shown by parallel activity in a double antibody enzyme-linked immunosorbent assay (ELISA). Although less sensitive, the RPLA method is easier to perform than the ELISA and counterimmunoelectrophoresis, both of which require special test reagents and equipment not generally available.

Clostridium perfringens food poisoning has remained the third leading cause of foodborne disease in the United States for the past several years. In 1981, there were 1,162 cases and 28 outbreaks reported to the Centers for Disease Control (2). Foodborne illness caused by *C. perfringens* results from the action of an enterotoxin produced in the intestine after sporulation of ingested vegetative cells (3). Although a variety of laboratory techniques have been used over the years to confirm *C. perfringens* as the specific cause of outbreaks (5-7), the concept of confirming outbreaks by detecting enterotoxin directly in the feces of patients has recently gained favor (1,8,13). This approach, however, has been of limited use because so few laboratories have suitable equipment and test reagents to examine stool specimens for enterotoxin. In confirming outbreaks, the limited availability of reagents and the well-known difficulties in obtaining good sporulation of *C. perfringens* on laboratory media are also responsible for preventing the routine testing of cultures implicated in food poisoning.

In 1983, we developed a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) similar to that described by Bartholomew et al. (1) for detection and quantitation of *C. perfringens* enterotoxin (CPE) in culture supernatant fluids and extracts from stools of food poisoning patients (13). While these studies were in prog-

ress, the Denka-Seiken Co., Tokyo, Japan, began to market a reversed passive latex agglutination (RPLA) test kit for perfringens enterotoxin (PET) (available in the USA from Oxoid, USA, Columbia, MD 21045), which promised to greatly simplify its identification and quantitation. We investigated the ability of the PET-RPLA Seiken test kit to identify and quantitate CPE in culture fluids and in extracts from feces of food poisoning patients.

Extracts from feces of 18 persons affected in six different food poisoning outbreaks and supernatant fluids from 40 *C. perfringens* cultures grown in modified AEA spore broth were tested for enterotoxin with the Seiken PET-RPLA test kit. The enterotoxin was quantitated and confirmed by the double antibody sandwich ELISA developed in our laboratory. The sensitivity and specificity of the test reagents supplied with the kits and their limits in actual use were determined.

MATERIALS AND METHODS

Culture supernatant fluids

Twenty enterotoxigenic *C. perfringens* cultures isolated from feces of patients who experienced diarrhea during one of seven different *C. perfringens* food poisoning outbreaks were cultured in modified AEA spore broth as described previously (5). To enhance sporulation and enterotoxin production, the cultures were heat-shocked and subcultured twice in fluid thioglycollate medium, as recommended by Duncan and Strong (4). After incubation for 24 h at 37°C, the cultures were examined microscopically for spores. Those in which good sporulation (20%) was observed were centrifuged for 30 min at 25,000 × g and the cell-free supernatant fluid was collected for toxin testing. Twenty nonenterotoxigenic *C. perfringens* cultures isolated from normal stools were cultured in the same manner and used as controls.

Fecal extracts

Fecal extracts were prepared by suspending 0.2 ml of liquefied stool in 1.8 ml of phosphate-buffered saline solution containing 0.01% merthiolate (PBSM) by using a Vortex mixer and centrifuging, usually for 30 min, at 15,000 × g to remove

insoluble solids. The clarified supernatant fluid was aspirated and tested for CPE without further treatment. To demonstrate that the extraction procedure effectively recovered low levels of enterotoxin, we added purified enterotoxin to four enterotoxin-negative stools at levels of 25, 50 and 250 ng/g and measured the amount of enterotoxin in extracts prepared as described above and in identical portions diluted 1:5 with PBSM.

RPLA test

Culture supernatant fluids or fecal extracts were tested for enterotoxin by diluting the test portion in PBS plus 0.5% (wt/vol) bovine serum albumin (BSA) and mixing 25 μ l with an equal volume of control latex or latex sensitized with antibody to CPE purified by affinity chromatography in individual wells of a V-type microtiter plate. The suspensions were mixed well on a micromixer, and the plate was incubated overnight at room temperature in a humid chamber. Test results were read with a test reading mirror (Dynatech Laboratories, Alexandria, VA). Purified CPE solutions of known concentration were diluted in PBS plus 0.5% (wt/vol) BSA and used as controls and to ascertain the sensitivity of the reagents supplied with the RPLA test kits.

ELISA test

Culture supernatant fluids and fecal extracts were tested for enterotoxin by the procedures of Wimsatt et al. (13). Purified CPE and antibody conjugated with horseradish peroxidase were supplied by John Wimsatt, Food and Drug Administration (FDA), Cincinnati, OH. Rabbit anti-CPE serum was obtained from Dr. Gerald Stelma, FDA, Cincinnati, OH. Details of reagents and procedures for the double antibody ELISA for CPE have been described by Shah et al. (10).

RESULTS AND DISCUSSION

Culture supernatant fluids

Table 1 shows the amounts of enterotoxin found in culture supernatant fluids of 20 different *C. perfringens* cultures by the RPLA test kit and the corresponding amounts as measured by the double antibody sandwich ELISA. Undiluted supernatant fluids from 20 enterotoxin-negative strains were also tested for CPE with negative results (data not shown). The data in Table 1 show by the RPLA that enterotoxin was present in culture fluids at levels ranging from 0.13 to 100 μ g/ml and could be roughly quantitated by this method. Corresponding activity in the double antibody sandwich ELISA was specific for CPE. The sensitivity of the RPLA test kits for identifying and quantitating enterotoxin in diluted culture supernatant fluids was approximately 3 ng/ml, as stated by the manufacturer. This compares favorably with the 1 ng/ml limit of ELISAs (1,8,9,13). Although the ELISA tests are undoubtedly more sensitive, such differences are unlikely to be of practical importance because the amount of enterotoxin produced by most *C. perfringens* strains is usually many times greater (11,12).

Fecal extracts

Table 2 shows the results obtained with fecal extracts from three patients affected in each of six different food poisoning outbreaks. Enterotoxin levels from 25 ng/g to 100 μ g/g were determined by both the RPLA and

TABLE 1. Comparison of the RPLA test with the direct double sandwich ELISA for identifying and quantitating *C. perfringens* enterotoxin in culture supernatant fluids^a.

| Culture | Spore count/ml of culture ^b ($\times 10^6$) | Minimum concentration of CPE (μ g/ml) ^c | |
|---------|--|--|-------|
| | | RPLA | ELISA |
| 1 | 23 | 25 | 40 |
| 2 | 44 | 50 | 60 |
| 3 | 4 | 12.5 | 9 |
| 4 | 17 | 25 | 30 |
| 5 | 2 | 1.25 | 2 |
| 6 | 5 | 12.5 | 22 |
| 7 | 18 | 25 | 38 |
| 8 | 32 | 50 | 13 |
| 9 | 0.2 | 0.13 | 0.03 |
| 10 | 16.3 | 12.5 | 10 |
| 11 | 9 | 25 | 18 |
| 12 | 45 | 50 | 24 |
| 13 | 3.8 | 12.5 | 40 |
| 14 | 52 | 50 | 38 |
| 15 | 15 | 25 | 16 |
| 16 | 17 | 12.5 | 23 |
| 17 | 6.4 | 25 | 49 |
| 18 | 24 | 6.3 | 19 |
| 19 | 30 | 25 | 8 |
| 20 | 1.1 | 3.1 | 9 |

^aIsolates from feces of food poisoning patients were grown in modified AEA spore broth at 37°C for 24 h.

^bAfter heating for 20 min at 75°C. Counts were made in tryptose-sulfite-cycloserine agar.

^cEstimated. Two-fold differences in enterotoxin concentration could not be determined reliably by RPLA.

ELISA. In five instances, it was necessary to dilute the fecal extract 1:60 or more before enterotoxin could be quantitated because of interference from other components of the extract. This difficulty was similar to that also noted by McClane and Strouse (8) and Wimsatt et al. (13). To explore this problem further and to determine the practical sensitivity limits of the RPLA test in examining stools, we added low levels (0.025 to 0.25 μ g/g) of purified enterotoxin to enterotoxin-negative stools of four healthy persons and quantitated the enterotoxin recovered. Table 3 shows that CPE could be determined at the lowest level added (0.025 μ g/g) in two of the four test specimens, but not in the other two. With the latter two specimens, it was necessary to dilute the fecal extract to 1:40 or higher to quantitate the enterotoxin without interference. These data indicate that, at least in some instances, the practical sensitivity limit of the RPLA test for examining outbreak stools is in the 50 to 100 ng/g range. This is about the same amount that can be reliably determined in stools by the double antibody sandwich ELISA and other sensitive serological methods (1,8,11-13).

CONCLUSIONS

The RPLA is a practical method for identifying and quantitating CPE in culture supernatant fluids and in ex-

TABLE 2. Comparison of RPLA with direct ELISA for detection and quantitation of *C. perfringens* enterotoxin in stools of food poisoning patients.^a

| Patient ^b | Enterotoxin (µg/g) ^c | |
|--------------------------------|---------------------------------|-------------------|
| | RPLA | ELISA |
| 1 (1) | 12.5 | 22 |
| 2 (1) | 12.5 | 16 |
| 3 (1) | 25 | 25 |
| 4 (2) | 100 | 90 |
| 5 (2) | 25 | 19 ^d |
| 6 (2) | 6.25 | 8 |
| 7 (3) | 12.5 ^d | 50 |
| 8 (3) | 6.25 | 5-10 |
| 9 (3) | 50 | 38 |
| 10 (4) | 25 | 19 |
| 11 (4) | 3.1 | 14 |
| 12 (4) | 0.12 | 0.6 |
| 13 (5) | 0.5 | 0.13 ^d |
| 14 (5) | 0.625 | 0.32 |
| 15 (5) | 1.25 | 1 |
| 16 (6) | 0.025 ^d | 0.03 ^d |
| 17 (6) | 0.005 ^d | 0.01 |
| 18 (6) | ND ^e | 0.003 |
| Negative controls ^f | <0.003 | <0.0006 |

^aSpecimens collected 12 to 36 h after onset of symptoms.
^bSimilar results were obtained with two additional specimens from each outbreak with the RPLA or ELISA test only. Outbreak number appears in parentheses.
^cEstimated. Two-fold differences in enterotoxin concentration could not be determined reliably by RPLA.
^dInterference with enterotoxin detection was noted with sample dilutions <1:60.
^eNot determined. Amount present could not be ascertained because of interference.
^fStools from six patients with diarrhea not caused by *C. perfringens*.

tracts from stools of food poisoning patients. The availability of a test kit such as the PET-RPLA (Denka-Seiken Co.) should make possible the routine testing of outbreak stools for enterotoxin and the examination of *C. perfringens* strains implicated in outbreaks. This test kit met our requirements by consistently and accurately identifying and quantitating CPE in both sample types with a minimum of time and effort. Our results confirmed that the sensitivity of the RPLA test kit was about 3 ng/ml of test portion (except with fecal extracts that contained interfering substances), as stated by the manufacturer of the kits. This sensitivity did not change significantly with kits that were stored up to 3 months at 4°C.

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TABLE 3. Detection and quantitation of low levels of *C. perfringens* enterotoxin of known concentration added to human feces using RPLA.

| Specimen | Enterotoxin (µg/g of prepared fecal suspension) ^a | | Dilution required ^c for toxin specificity |
|----------|--|-----------------|--|
| | Enterotoxin detected (µg/g) ^b | | |
| 1 | 0.25 | 0.25 | 1:10 |
| 2 | 0.25 | 0.25 | 1:60 |
| 3 | 0.25 | 0.25 | 1:10 |
| 4 | 0.25 | 0.25 | 1:40 |
| 1 | 0.05 | 0.05 | 1:10 |
| 2 | 0.05 | 0.03 | 1:60 |
| 3 | 0.05 | 0.05 | 1:10 |
| 4 | 0.05 | ND ^d | 1:40 |
| 1 | 0.025 | 0.0125 | 1:5 |
| 2 | 0.025 | ND | >1:20 |
| 3 | 0.025 | 0.025 | 1:5 |
| 4 | 0.025 | ND | >1:20 |

^aFeces were diluted 1:1 in PBS and indicated amounts of purified enterotoxin were added.
^bSensitized latex was supplied by Denka-Seiken. Sensitivity with purified enterotoxin was 3 ng/ml.
^cDilution of extract in PBS in which enterotoxin could be detected without interference.
^dNot detected because fecal extract produced nonspecific reactions.

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