Detection of *Clostridium perfringens* Enterotoxin in Human Fecal Samples and Anti-Enterotoxin in Sera

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By using counterimmunoelectrophoresis (CIEP), *Clostridium perfringens* enterotoxin was successfully demonstrated in fecal samples collected within 1 day of attack from sick individuals involved in a bacteriologically and epidemiologically proven outbreak of *C. perfringens* food poisoning. In contrast, enterotoxin was not demonstrable in fecal samples of apparently healthy individuals both at high- and low-risk exposure to the organism and enterotoxin or in fecal samples collected 4 to 5 days after a food poisoning outbreak. A 100% prevalence of *C. perfringens* anti-enterotoxin in sera of human volunteers at high- as well as low-risk exposure to the organism and enterotoxin was recorded with CIEP.

Clostridium perfringens type A food poisoning is very important from the public health point of view. The number of people usually involved in a food poisoning outbreak due to *C. perfringens* is so large that it immediately becomes obvious that an outbreak has occurred. The symptoms may vary from mild to severe diarrhea with variable incubation periods, i.e., from less than 5 to 6 h to 8 to 22 h, depending upon the environmental factors and the food substrate. It is well known that the enterotoxin responsible for human food poisoning is only produced during sporulation and not during vegetative growth of *C. perfringens* (3-5).

A suspected *C. perfringens* food poisoning outbreak can be confirmed only after prolonged bacteriological examination of the suspected food and fecal samples from the affected individuals. A quick conclusion regarding involvement of *C. perfringens* in an outbreak is desirable. To our knowledge there is only one report (1) in the literature in which it was possible to detect *C. perfringens* enterotoxin in stools of victims in an epidemiologically and bacteriologically proven food poisoning outbreak, and this was done by using a reversed passive hemagglutination (RPHA) test with 100% sensitivity. However, in our experience the reproducibility of the RPHA test is variable (6; Naik and Duncan, unpublished data). Uemura et al. (12) used the RPHA test to demonstrate enterotoxin in vomitus and fecal samples of monkeys; to avoid nonspecific hemagglutination, the vomit and fecal samples

were absorbed beforehand with nonsensitized sheep erythrocytes. Recently (1), sheep erythrocytes fixed and sensitized with anti-enterotoxin immunoglobulin G (IgG) using glutaraldehyde were found to be more stable for enterotoxin detection than the bindiazobenzidine used by other authors (13) for coupling. Fluorescent-antibody (FA) staining (10) of many fecal samples from apparently healthy individuals for enterotoxin-producing *C. perfringens* cells was hard to interpret because of a debris with self-fluorescence. In addition, the validity of the FA technique in an actual *C. perfringens* food poisoning outbreak has not been tested.

Uemura et al. (11) used the passive hemagglutination (PHA) test to detect anti-enterotoxin in human sera. Dowell et al. (1) reported that glutaraldehyde was successful for sensitizing human erythrocytes (O group, Rh negative) with purified enterotoxin for the assay of anti-enterotoxin in sera. Also, Torres-Anjel and Riemann (9) used the FA inhibition technique to determine serum antibodies against *C. perfringens* type A enterotoxin.

The present report demonstrates the usefulness of a very simple counterimmunoelectrophoresis (CIEP) procedure for assay of the *C. perfringens* enterotoxin in fecal samples of individuals involved in an epidemiologically and bacteriologically proven outbreak of *C. perfringens* food poisoning. The possibilities of using this technique to quickly demonstrate and titrate antibodies in human sera against *C. perfringens* enterotoxin are discussed.

MATERIALS AND METHODS

Human fecal samples of individuals involved in

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presumed *C. perfringens* food poisoning outbreaks and isolated cultures from outbreaks were obtained from the State Laboratory of Hygiene. In outbreak no. 1, the number of people involved was 160 to 170. The incubation period ranged from 4 to 15 h, with symptoms of mild abdominal cramps and diarrhea occurring on 15 February 1977 and persisting for less than 1 day. Fecal samples were obtained within 1 day after the appearance of symptoms. Two *C. perfringens* cultures were isolated from each of eight victim's stools without a heat selection process, and a third culture was isolated after heat shock of the sample at 75°C for 20 min. Serotyping of the isolates revealed a common Hobbs' serological type 1 in six of the eight victims' stools.

In outbreak no. 2, four out of six individuals at risk experienced cramps, diarrhea, nausea, and chills 12 to 13 h after eating in a restaurant. No food was available for testing. However, stool specimens were obtained from all six individuals 4 to 5 days after the meal. Cultures of *C. perfringens* were isolated from the stool specimens as above. Hobbs' serological type 9 was common to two of the individuals, and type 1 was common to the remaining two individuals.

In addition to the above, fecal samples were obtained from seven apparently healthy individuals in our laboratory who were working daily with *C. perfringens* cultures and individuals in other laboratories who were not exposed to such cultures.

Assay of enterotoxin in fecal samples. Fecal samples were diluted 1:2 with 0.85% saline in demineralized distilled water to make a very thick suspension and were then centrifuged at 2,000 rpm for 20 min at room temperature. The supernatant fluid was collected and diluted twofold with microtiter titrators (Cooke Engineering Co., Alexandria, Va.). Each sample dilution was tested for enterotoxin by the CIEP procedure as described by Naik and Duncan (6), using anti-enterotoxin raised in a rabbit against purified enterotoxin (8).

Screening of cultures isolated from food poisoning outbreaks for their enterotoxin-reducing capabilities. A 0.1-ml amount of *C. perfringens* culture was transferred from cooked-meat medium (Difco, Detroit, Mich.) to 10 ml of Duncan and Strong (DS) sporulation medium (2) and was allowed to grow for 18 to 20 h at 37°C. Then 0.1 ml of the DS culture was transferred to 10 ml of fluid thioglycolate medium (BBL, Cockeysville, Md.), heat shocked at 75°C for 20 min, and incubated at 37°C for 18 to 20 h, and the entire culture was inoculated into 10 ml of DS medium. This was incubated at 37°C for 24 h. Assay of enterotoxin in the DS culture supernatant fluid was done by the CIEP assay.

Erythematous activity in culture supernatant. Ten milliliters of a 24-h-old DS culture was removed and centrifuged at 2,000 rpm for 20 min. The culture supernatant fluid was tested for enterotoxin by the erythematous assay in guinea pig skin as previously described (7).

Assay of *C. perfringens* type A anti-enterotoxin titer in human sera. Nineteen sera were collected from human volunteers working at the Food Research Institute. The group was comprised of persons working directly with *C. perfringens* and its en-

terotoxin (high risk) as well as from those who had no opportunity to come in contact with laboratory *C. perfringens* cultures or their enterotoxin (low risk). Some of the sera were fractionated at 35% ammonium sulfate as well as by QAE-Sephadex A-50 chromatography (Pharmacia Fine Chemicals, Technical Data Sheet 30M-4/71-UP). Whole sera as well as the fractionated sera were diluted twofold with 0.025 M sodium phosphate-buffered saline, pH 7.2. The anti-enterotoxin titer was determined by CIEP and using a constant toxin concentration of 1.05 µg/10 µl in 0.025 M phosphate-buffered saline, pH 7.2.

RESULTS AND DISCUSSION

Table 1 shows the titer of *C. perfringens* type A enterotoxin in fecal samples and sporulating cultures of fecal *C. perfringens* isolates from individuals involved in food poisoning outbreak no. 1. The CIEP titer of enterotoxin in the feces ranged from 1:32 to 1:128. All but one *C. perfringens* culture isolated from the feces were positive for enterotoxin production. Fecal samples examined from seven people working directly in our laboratory with *C. perfringens* cultures and their enterotoxin and from three people working in a different laboratory on a different floor were found to be negative for the enterotoxin.

In outbreak no. 2, in which four out of six

TABLE 1. Enterotoxin detection in fecal samples and cultures from feces of eight individuals with *C. perfringens* food poisoning^a

Stool	Enterotoxin CIEP titer	Culture	Enterotoxin in 24-h DS culture supernatant
1	1:64	1-1	+
		1-2	+
		1-3	+
2	—	2-1	—
		2-2	+
		2-3	+
3	1:256	3-1	+
		3-2	+
		3-3	+
4	1:64	4-1	+
		4-2	+
		4-3	+
5	1:64	5-1	+
		5-2	+
		5-3	+
6	1:32	6-1	+
		6-2	+
		6-3	+
7	1:128	7-1	+
		7-2	+
		7-3	+
8	1:128	8-1	+
		8-2	+
		8-3	+

^a Fecal samples were collected within 1 day after appearance of food poisoning symptoms. All individuals had food poisoning symptoms.

individuals at risk had symptoms, enterotoxin was not detected in the feces of any of the six subjects by CIEP (Table 2). However, five of the six fecal isolates of *C. perfringens* were enterotoxin producers. The reason for not detecting enterotoxin in the feces of the individuals with symptoms in this outbreak could be due to the fact that either there was too little enterotoxin for detection or, more likely, the enterotoxin had already been completely eliminated from the intestinal tract, since the fecal samples were collected 4 to 5 days after the appearance of symptoms. Dowell et al. (1) reported that stool samples collected from individuals after a *C. perfringens* food poisoning attack did not have enterotoxin as measured by RPHA. They also concluded that enterotoxin disappears quickly even from positive stools just after the disappearance of the symptoms. Therefore, use of the CIEP procedure to quickly demonstrate enterotoxin in feces and confirm *C. perfringens* food poisoning should be done as soon after appearance of symptoms as possible. At this point, it is not clear how long the toxin continues to be present in the feces after food poisoning symptoms have disappeared. This would need to be determined in several actual outbreaks.

Enterotoxin production by cultures of *C. perfringens* isolated from both outbreaks reported here (Tables 1 and 2) clearly indicates that enterotoxin could be easily demonstrated after 24-h growth of the isolate in DS medium. Thus, the CIEP procedure is useful to quickly demonstrate the enterotoxin-producing capabilities of a suspected culture after 24 h of growth in DS medium at 37°C.

Table 3 shows the titer of *C. perfringens* type A anti-enterotoxin in 10 human sera from laboratory people at high risk as well as from those who were not working with *C. perfringens* or its enterotoxin.

Of 19 sera examined, all were found to possess serum antibodies against type A *C. perfringens*

TABLE 2. Examination of fecal samples and *C. perfringens* culture isolates for enterotoxin using CIEP^a

Stool	Symptoms	Enterotoxin CIEP titer in fecal samples	Enterotoxin in 24-h DS culture supernatant fluid
1	Not ill	-	+
2	Not ill	-	+
3	Ill	-	-
4	Ill	-	+
5	Ill	-	+
6	Ill	-	+

^a Fecal samples were collected 4 to 5 days after appearance of food poisoning symptoms.

TABLE 3. Titer of type A *C. perfringens* anti-enterotoxin in human sera

No. of individuals with the indicated serum titer	Reciprocal of CIEP titer against 1.05 µg of enterotoxin in 0.025 M PBS, pH 7.2 ^a
1	1:2
3	1:4
6	1:8
8	1:16
1	1:32

^a PBS, Phosphate-buffered saline.

enterotoxin, with titers ranging from 1:2 to 1:32. The degree of known exposure to the organism or enterotoxin appeared not to influence the serum titer. In fact, the highest titer (1:32) was in an individual not working with the organism or toxin. Dowell et al. (1) also found 100% positive serum samples for anti-enterotoxin by using a PHA assay. Uemura et al. (11) reported the incidence of anti-enterotoxin to be 82% in Brazilian and 65% in American individuals. However, in their study, the sera were diluted initially 1:80 and were not designated positive with a titer less than 1:160.

In the present study, fractionation of sera with 35% ammonium sulfate and separation of the IgG fraction by ion-exchange chromatography on QAE-Sephadex A-50 did not increase the titer of the original sera, but in some cases it did reduce the CIEP titer of the sera. The results are not surprising, since the yield of IgG using a QAE-Sephadex A-50 column is only about 70% (Pharmacia Fine Chemicals, Technical Data Sheet 30M-4/71-UP) and varies depending upon the sera. Torres-Anjel and Riemann (9) reported that diethylaminoethyl-Sephadex A-50 fractionation of sera increased the sharpness of the end point in an FA inhibition technique for the demonstration of anti-enterotoxin in some but not all human sera. Thus, fractionation of sera for anti-enterotoxin demonstration appears unnecessary if the CIEP procedure for titer determination is used.

The high prevalence of *C. perfringens* anti-enterotoxin in human serum suggests that individual exposure to *C. perfringens* food poisoning is common or that the indigenous intestinal *C. perfringens* flora produces sufficient toxin for an immune response without symptoms of food poisoning occurring. It would be interesting to know whether a rise in anti-enterotoxin titer occurs after an attack of *C. perfringens* type A food poisoning. Dowell et al. (1) recorded that the log 2 PHA anti-enterotoxin titer of human serum samples collected after a food poisoning outbreak due to *C. perfringens* varied from 3 to 11, but the titers before the outbreak were unknown.

It is interesting to note that the sera collected before immunizing rabbits with enterotoxin are completely negative for anti-enterotoxin. Obviously, exposure of these animals to enterotoxin has been different from that of humans. This is true even though *C. perfringens* can be isolated from normal rabbit fecal material.

In summary, CIEP appears to be a useful procedure for the epidemiological investigation of *C. perfringens* food poisoning outbreaks. Confirmation of *C. perfringens* food poisoning may be aided by demonstrating enterotoxin in fecal samples of victims by CIEP. Further study should be done to determine the optimal time for collection of such samples from patients for toxin analysis.

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