Rapid Detection of Clostridium botulinum Toxin by Capillary Tube Diffusion

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A micro capillary agar-gel diffusion system for the detection of botulinal toxin in foods and cultures was developed and evaluated. Toxins types A, B, and E, produced in culture broth with and without added trypsin, and type E toxin, produced in inoculated canned clams, were tested with this system and with the mouse bioassay procedure. With nontrypsinized toxin, the capillary diffusion system detected as little as 100 minimal lethal doses (MLD) per ml but was effective only at higher levels, 10^4 to 1.5 × 10^5 MLD/ml, when used with trypsinized toxin. The inability to detect lower levels of trypsinized toxin was due to thioglycolate present in the medium used to produce toxin. Evidently, trypsinization of toxin produces polypeptides still held together by disulfide bonds. Cleavage of these bonds by reduction with thioglycolate reduces the sensitivity of the capillary method. Trypsinized toxin produced in broth without thioglycolate was detected as readily as nontrypsinized toxin. Toxic was detected in canned clams containing as low as 100 MLD/ml. No cross-reactions were observed with type E toxin and types A and B antitoxins. Extensive studies using the capillary method for detecting types A and B toxins were not performed; however, a suspected sample of commercially canned mushrooms gave a positive type B reaction but not a type A reaction. This typing was confirmed later by the mouse bioassay. Toxic was present at a level of 100 MLD/ml. The procedure developed may prove useful as a rapid screening method for the detection of botulinal toxin in foods, with final identification made by using the mouse bioassay.

The most frequently used procedure for detecting toxin from Clostridium botulinum is to inject dilutions of suspected extracts intraperitoneally into mice and determine the minimal lethal dose (MLD) per milliliter or the lethal dose for 50% of the population (LD50) per milliliter (14). Various immunological procedures have been used for detection of botulin toxins in vitro. Johnson et al. (8) developed a hemagglutination procedure sensitive enough to detect one mean lethal dose mouse unit of botulin toxin. Serological reactivity of several other toxins can be measured by the Ouchterlony gel-diffusion plate procedure (9, 20). Small quantities of other proteinaceous toxins, such as staphylococcal enterotoxins in foods, can be assayed by immunodiffusion methods (3, 13). A slide technique using gel diffusion (Ouchterlony) to detect the toxins of Clostridium botulinum in foods was described by Vermilyea et al. (19). Although this method is useful, it is difficult to prepare and store slides for testing large numbers of samples. This paper describes an adaptation of Vermilyea's procedure that uses capillary tubes containing antitoxin in an agar support at one end and toxin sample at the other. This micro capillary agar-gel diffusion system was tested with type E toxin and compared with the mouse bioassay. (This paper is taken in part from a thesis submitted in partial fulfillment of the requirements for the M.S. degree from Wagner College, Staten Island, N.Y.)

MATERIALS AND METHODS

Production of type E toxin. Frozen spore suspensions of C. botulinum type E were obtained from the stock culture collection of the Microbiology Section, Food and Drug Administration, New York District, Brooklyn. Spores were diluted before use in Butterfield phosphate buffer, pH 7, to a concentration of 5 × 10^6 spores/ml. Spore numbers were determined by dilution plate counts on brain heart infusion agar (Difco) plus 0.1% sodium thioglycolate incubated anaerobically for 48 h at 35 C. For toxin production,
spores were inoculated into TPGY broth (5% tryptic soy [Difco], 5% peptone [Difco], 0.4% glucose, 2% yeast extract [Difco], and 0.1% sodium thioglycollate) as described by Schmidt et al. (17) and Lilly et al. (11). The medium was adjusted to pH 7.0 and dispensed in screw-capped tubes in 40-ml quantities before autoclaving. TPGY broth (0.1% filter-sterilized trypsin [Difco] added to tubes of TPGY broth after sterilization) was used for production of trypsinized toxin (7).

Tubes of TPGY and TPGY broth were inoculated with $5 \times 10^5$ spores and incubated at 30 C for periods ranging between 1 and 5 days to produce a range of toxin titers. Samples (30 ml) from each tube were centrifuged at 48,000 $\times g$ for 30 min at 4 C and filtered through sterile disposable filter units (0.45 $\mu$m; Naige-Sybron Corp., Rochester, N.Y.). The filtered broth was either assayed immediately or stored frozen to maintain toxin stability until analysis.

Botulinum type E toxin was also produced by inoculating 8-oz (about 240 ml) cans of minced clams, obtained commercially. The cans were autoclaved for 20 min at 121 C, and, after cooling, a hole approximately 1 mm in diameter was punctured aseptically in the top of each can. A 1-ml sample of a spore suspension ($5 \times 10^5$ spores/ml) was injected with a sterile syringe. Control cans were inoculated with 1 ml of Butterfield phosphate buffer, pH 7.0. The holes were sealed with solder, and the cans were incubated at 30 C for either 1, 2, 3, 4, or 5 days.

After incubation, the top of each can was washed with 95% ethanol and flamed before a hole, 5 cm in diameter, was cut with a bacteriological disk cutter. A 30-ml amount of clam juice was removed and centrifuged at 48,000 $\times g$ for 30 min at 4 C to eliminate food particles. The milky, opalescent supernatant liquid was filtered through presterilized filter units (0.45 $\mu$m). The filtered liquid was divided into two 15-ml portions. One portion was transferred to a centrifuge tube; 10 g of dry, powdered Sephadex G-25 Fine (Pharmacia, Fine Chemicals, Piscataway, N.J.) was added, and a semisolid slurry was formed. The tube was allowed to stand at room temperature for 3 h with mixing every half hour. The slurry was then centrifuged at 1,000 $\times g$ for 30 min at 4 C. The supernatant fluid (approximately 3 ml) was decanted and analyzed by the micro capillary agar-gel diffusion method. The remaining 15-ml portion was used for the mouse bioassay method.

Production of types A and B toxins. Frozen spore suspensions of botulinum types A and B were obtained from the culture collection of the Food and Drug Administration. A 1-ml amount of a standard dilution of each spore suspension ($5 \times 10^5$ spores) was inoculated into separate tubes containing 40 ml of TPGY broth. After incubation at 30 C for 5 days, 10 ml from each tube was removed and filtered through 0.45-$\mu$m presterilized disposable filters. One portion of the filtered broth was used to determine the mouse MLD. Ten-microliter samples were tested against the types A, B, and E antitoxin by the capillary diffusion system.

Determination of mouse MLD. Toxin titers of both trypsinized and untrypsinized type E toxin were determined by a mouse bioassay method similar to the procedure described by Dolman (6). Samples containing toxin were diluted in 10-fold increments with sterile gel-phosphate buffer, pH 6.2 (7). The buffer contained 0.2% gelatin (Difco) in 1% Na$_2$HPO$_4$, and the pH was adjusted with NaOH before sterilization. A 0.5-ml amount of each dilution was inoculated intraperitoneally to each of 10 Swiss-strain mice (18 to 24 g). As controls, 10 mice were inoculated with 0.5 ml of sterile buffer. Deaths were recorded at the end of 4 days and reported as MLD per milliliter.

Botulinal antitoxin. Dehydrated types A, B, and E antitoxins were obtained from the Center for Disease Control, Atlanta, Ga. The antitoxins, rehydrated as directed, contained 10 IU per ml as defined by Bowmer (1). Control mice inoculated with 0.5 ml of a 1:5 dilution of the appropriate antitoxins survived subsequent injection with toxin.

Micro capillary agar-gel diffusion technique. The Ouchterlony gel-diffusion system for the detection of botulinum toxin (4, 19) was modified for use with capillary tubes. The diffusion medium was 1% agar in borate saline buffer, pH 8.4, as described by Carpenter (2). Borate saline buffer was prepared as follows: 94 ml of 0.85% NaCl was added to 5 ml of an aqueous solution containing 0.62% boric acid, 0.95% sodium tetraborate, and 0.44% NaCl. One gram of Special Noble agar (Difco) was added and dissolved by boiling. After cooling, 1 ml of 1% Thimersol (City Chemical Co., New York, N.Y.) was added as preservative. The diffusion agar was dispensed in 10-ml quantities in screw-capped test tubes and stored at 4 C.

Melted agar was cooled to 40 C before use. A 3-ml amount of undiluted antitoxin was mixed with 2 ml of agar, and 10 $\mu$l of this mixture was added to one end of a glass capillary tube (4 cm long, 0.95-mm inner diameter, 1.35-mm outer diameter) by means of a 50-$\mu$l syringe (Hamilton) with a blunt-ended needle. The filled end of the tube was plugged by inserting it approximately 1 mm into a block of Sealease tube sealer and holder (Scientific Products). With a clean syringe, 10 $\mu$l of toxin was carefully added to the open end of the tube so as to avoid entrapment of bubbles. This was done by inserting the blunt needle into the tube until it nearly touched the solidified antitoxin-agar mixture in the other half of the tube. The needle was withdrawn slowly as the fluid was dispensed.

Tubes filled in this manner were placed horizontally in a petri dish containing moistened filter paper. Wooden applicator sticks were used to support the capillary tubes above the paper. The tubes were observed after incubation at 30 C from 3 to 24 h. During incubation, tubes with positive reactions developed a thin precipitin line within the antitoxin-agar mixture. This line was approximately 1 mm from the interphase with the toxin sample. Lines were easily visible under $\times 1.5$ magnification using reflected light against a dark background (see Fig. 1).

RESULTS
Detection of toxin in culture media. Table 1 shows the amounts of type E toxin produced in
tubes of TPGY and TPGYT broths after incubation for 1 to 5 days at 30 C. The toxin activity detected by the mouse bioassay ranged from 0 to 10^6 MLD/ml for nontrypsinized and 10^4 to 1.5 \times 10^7 MLD/ml for trypsinized toxin. These samples were assayed qualitatively for toxin by the micro capillary agar-gel diffusion procedure. Positive reactions consisting of a thin precipitin line were detected in all nontrypsinized samples including the 1-day sample, which did not contain a detectable amount of toxin on the basis of the mouse bioassay. With trypsinized toxin, only the two higher levels (1.5 \times 10^7 and 1.0 \times 10^6 MLD/ml) gave positive reactions in the capillary tubes. No precipitin lines were detected in the control tubes. All reactions were read between 3 and 24 h. These same toxin samples were stored at 4 C for 2 weeks and assayed daily by the micro capillary method, and in each case the results were as reported above. Photomicrographs of typical positive reactions at the various toxin levels for nontrypsinized toxin are shown in Fig. 1. The entire experiment was repeated beginning with freshly inoculated tubes of TPGY and TPGYT broths on two other occasions with exactly the same results.

It was difficult to understand why the capillary gel procedure was so much less sensitive in detecting trypsinized toxin than in detecting toxin produced in TPGY broth without trypsin, even recognizing that 1 MLD of trypsinized toxin represents much less antigenic protein than 1 MLD of nontrypsinized toxin (because trypsinization makes the protein more biologically active). No report in the literature indicated that trypsinization greatly affects serological reactivity. Sakaguchi and Sakaguchi (16) showed that activation with trypsin at pH 6.0 did not appreciably affect the molecular size of the toxin as determined by Sephadex gel chromatography and analytical ultracentrifugation. Recently, however, DasGupta and Sugiyama (5) found that trypsin-activated toxin separated into at least two smaller polypeptides with molecular weights of 50,000 and 102,000 when reduced with \( \beta \)-mercaptoethanol before polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The nontrypsinized toxin was unaffected by this reduction. Presumably, trypsinization cleaves the polypeptide chain into two smaller chains still held together by disulfide linkages unless these are also cleaved by reduction. This suggested that the reducing agent, sodium thioglycolate, used in the present work as an oxygen scavenger in the broth wherein toxin was produced, could affect detection of the trypsinized toxin in the capillary gel procedure by reducing disulfide linkages and allowing at least partial loss of antigenically active configuration.

This was confirmed in the following experiment in which trypsinized and nontrypsinized toxins were produced both in the presence and absence of sodium thioglycolate. Tubes of TPGY and TPGYT broth with and without 0.1% thioglycolate were autoclaved, cooled, and inoculated immediately with 0.1 ml of a 24-h broth culture of C. botulinum type E. After incubation for 3 days at 30 C, serial dilutions up to 10^-7 were prepared and assayed by the capillary gel procedure. In the absence of thioglycolate, precipitin lines formed with all dilutions of nontrypsinized toxin up to 10^-6 and with trypsinized toxin up to 10^-3. Nontrypsinized toxin produced in the presence of thioglycolate tested positive up to a dilution of 10^-3, but no visible precipitin lines were observed with samples from any dilution with the trypsinized toxin from broth with thioglycolate. Lower dilutions of trypsinized toxin formed in the presence of thioglycolate had a slight cloudiness distributed throughout the antitoxin-gel section of the capillary tube.

### Detection of toxin in canned clams.

In preliminary experiments, toxin in filtrates from canned minced clams was assayed directly (not treated with Sephadex) by the micro capillary agar-gel diffusion procedure with negative results. It was found that clam juice samples concentrated by treatment with Sephadex, as described in Materials and Methods, gave positive reactions by the capillary diffusion method. The concentration step was used for the capil-

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**Table 1. Comparison of the mouse bioassay and the micro capillary gel diffusion procedures for detecting toxin in TPGY (trypsinized) and TPGY (nontrypsinized) broths incubated for various times**

| Day | TPGY | TPGYT |
|-----|------|-------|
|     | (MLD/ml) | Micro capillary method | (MLD/ml) | Micro capillary method |
| 0^c | 0    |     | 0    |     |
| 1   | 10^-a|     | 0    | +  |
| 2   | 10^-a|     | 10^-u| +  |
| 3   | 10^-u|     | 10^-u| +  |
| 4   | 10^-v|     | 10^-v| +  |
| 5   | 1.5 \times 10^-v|     | 10^-v| +  |

^a Average of three inoculated tubes.
^b + , Visible precipitin line; −, no visible precipitin line.
^c Uninoculated 0-day control.
Fig. 1. Photomicrographs (×30) of capillary tubes containing 10 μlrites of type E toxin from TPGY broth showing typical reactions with the agar-gel diffusion system. The toxin titers in the preparation were as follows: A, 10⁶; B, 10⁴; C, 10³; D, 10²; E, 0 MLD/ml; F, uninoculated control. A through D show positive reactions. E and F are negative.

DISCUSSION

Botulism poisoning can be due to the ingestion of an underprocessed or improperly preserved low-acid food containing C. botulinum toxin. Until recently, the mouse bioassay was the only valid technique for the detection and identification of botulinic toxin. The procedure involves the intraperitoneal injection of products into mice. When deaths occur, the lethal factor must be shown to be neutralized with antitoxin to one of the botulinic toxin types. Injection of products directly into mice sometimes leads to "nonspecific" deaths due to properties of the product itself or bacterial products other than botulinic toxin. Deaths can also be caused by piercing vital organs during inoculation (12, 18).
The capillary method detected toxin in inoculated cans at levels corresponding to $1 \times 10^3$ to $1.5 \times 10^4$ MLD/ml in the mouse bioassay provided that the clam juice was treated with Sephadex before use. No toxin was detected in clam juice samples in any range which were not treated with Sephadex before assay by the capillary diffusion system. Treatment with Sephadex concentrated the sample and removed low-molecular-weight materials and, in addition, eliminated part of the opalescence which interfered with visualization of the precipitin line formed in positive tests.

Both trypsinized and nontrypsinized toxins produced in the absence of sodium thioglycolate were readily detected by the capillary diffusion system without concentration of the culture broths. Trypsinized toxin formed in the presence of thioglycolate was not easily detected, presumably because lower-molecular-weight polypeptides formed by the reduction of disulfide bonds either diffused too rapidly or were nonreactive with the antitoxin used. An alternative but less likely explanation is that trypsin-thioglycolate present in capillary tubes has an affect on the antitoxin which decreases the sensitivity of the precipitin reaction. Presumably, naturally activated toxin would readily be detected by the capillary procedure providing reducing agents are not present at levels comparable to 0.1% thioglycolate. In any event, the results show that treatment with trypsin is not necessary, since low levels of untrypsinized toxin were readily detectable.

The micro capillary agar-gel diffusion method has not been compared directly with the slide modification of the Ouchterlony gel diffusion technique described by Vermilyea et al. (19). According to these authors, the slide technique takes 24 to 48 h for visualization of precipitin lines, and cross-reactions between toxin types can occur. The micro capillary system takes from 3 to 24 h, and no cross-reactions were observed.

The micro capillary agar-gel diffusion system appears to have several advantages over the mouse bioassay. Capillary tubes are readily available and are easily filled with antitoxin and sample. Tubes with antitoxin have been stored for over 2 weeks at 4°C without affecting the assay. False positive results are reduced, since the problem of nonspecific mouse deaths due to “protein shock” or through human error in intraperitoneal inoculation are eliminated. In the present study, no false positive results were observed; however, a greater variety and number of contaminated foods must be examined for toxin before this point can be confirmed.

According to Kitamura et al. (10) both trypsinized and nontrypsinized type E toxin are complexes composed of toxigenic (Ec) and nontoxigenic (Eb) components. Evidently antitoxin is a mixture of at least two antibodies specific for one or the other of these components (15). Conceivably, false positives could be produced by the capillary gel procedure if only Eb components were present in a sample. This would mean that the usefulness of the capillary method would be limited to a screening of suspected samples followed by confirmation of all positives by the mouse bioassay.

One point must be made in evaluating the sensitivity of the capillary procedure in terms of the mouse bioassay. Although it was shown that the capillary system can detect as little as 100 MLD/ml of nontrypsinized toxin (Table 1), this may leave a false impression as to the actual sensitivity of the method. It is known that ingested toxin is activated in vivo. Therefore, perhaps a more realistic expression of sensitivity would be in terms of the MLD per milliliter as assayed in mice by using trypsin-activated toxin.

In the past 2 years, the New York District office of the Food and Drug Administration has analyzed over 4,000 samples of canned foods for botulinum toxin. Although confirmed positives are rarely encountered, at least 10 mice are used for each sample. Recently a sample of canned mushrooms was found to be toxic to mice. A sample of centrifuged liquid from this suspected product was examined by an analyst trained in the use of the capillary diffusion procedure. Since the test material was clear liquid, it was analyzed directly without Sephadex treatment. After 15 h, the tubes were examined, and a strong positive reaction for type B but not type

### Table 2. Mouse MLD levels of toxin detected in canned minced clams incubated for various times

| Day | MLD/ml | Micro capillary method |
|-----|--------|------------------------|
| 0   | 0      | -                      |
| 1   | $10^3$ | -                      |
| 2   | $10^3$ | +                      |
| 3   | $10^3$ | +                      |
| 4   | $10^3$ | +                      |
| 5   | $1.5 \times 10^4$ | +            |

*a* Toxin assayed without Sephadex treatment.

*b* Toxin concentrated with Sephadex G-25 before assay. Symbols: +, visible precipitin line; -, no visible precipitin line.

*c* Uninoculated 0-day control.
A toxin was observed. This typing was later confirmed by the mouse bioassay procedure at a titer of 100 MLD/ml. At present it is not possible to quantitate toxin titers by the capillary procedure, but it appears that the technique may be useful as a rapid, inexpensive screening procedure.

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LITERATURE CITED

1. Bowmer, E. J. 1963. Preparation and assay of the international standards for Clastidium types A, B, C, D and E antitoxins. Bull. W.H.O. 29:707-709.
2. Carpenter, P. L. 1968. Immunology and serology. W. B. Saunders Co., Philadelphia.
3. Casman, E. P., and R. W. Bennett. 1965. Detection of staphylococcal enterotoxin in foods. Appl. Microbiol. 13:181-189.
4. Crowle, A. J. 1958. A simplified microdouble diffusion agar precipitin technique. J. Lab. Clin. Med. 52:784-787.
5. DasGupta, B. R., and H. Sugiyama. 1972. A common subunit structure in Clostridium botulinum type A, B and E toxin. Biochem. Biophys. Res. Commun. 48:108-112.
6. Dolman, C. E. 1964. Growth and metabolic activities of Clostridium botulinum types. Public Health Service Publ. 99-FP-1, 43-68. Washington, D.C.
7. Duff, J. Y., G. G. Wright, and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. J. Bacteriol. 72:455-460.
8. Johnson, H. M., K. Brenner, R. Angelotti, and H. E. Hall. 1966. Serological studies on types A, B and E botulinum toxins by passive hemmaglutination and bentonite flocculation. J. Bacteriol. 91:967-974.
9. Kabat, C. A. 1961. Experimental immunochromatography. Charles C Thomas, Publishers, Springfield.
10. Kitamura, M., S. Sakaguchi, and G. Sakaguchi, 1968. Purification and some properties of Clostridium botulinum type E toxin. Biochim. Biophys. Acta 102:207-217.
11. Lilly, T., Jr., S. M. Harmon, D. A. Kautter, H. M. Solomon, and R. K. Lynt, Jr. 1971. An improved medium for detection of Clostridium botulinum type E. J. Milk Food Technol. 34:492-497.
12. Minor, N. A., J. Koehler, and L. Greenway. 1969. Intraperitoneal injection of mice. Appl. Microbiol. 17:250-251.
13. Reed, R. B., J. Bradshaw, W. L. Pritchard, and L. A. Blank. 1965. In-vitro assay of staphylococcal enterotoxins A and B from milk. J. Dairy Sci. 48:420-424.
14. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493-497.
15. Sakaguchi, G. 1968. Purification and molecular dissociation of Clostridium botulinum type E toxin. p. 341-347. Proc. First U.S.-Japan Conf. Toxic Micro-organisms.
16. Sakaguchi, G., and S. Sakaguchi. 1966. Some observation on activation of Clostridium botulinum type E toxin by trypsin. Botulism 1966. Proc. Fifth Int. Symp. Food Microbiol., Moscow.
17. Schmidt, C. F., W. K. Nark, and R. V. Lechovick. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation and radiation resistance of spores of Clostridium botulinum type E. J. Food Sci. 27:77-84.
18. Steward, J. P., E. P. Ornelas, K. D. Beernick, and W. H. Northway. 1968. Errors in the technique of intraperitoneal injection of mice. Appl. Microbiol. 16:1418-1419.
19. Vermilyea, B. L., H. W. Walker, and J. C. Ayres. 1968. Detection of botulinum toxins by immunodiffusion. Appl. Microbiol. 16:21-24.
20. Wadsworth, C. 1957. A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allergy Appl. Immunol. 10:335-360.