Immunodiffusion Method for Detection of Type A Clostridium botulinum

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Received 15 June 1981/ Accepted 20 August 1981

A simple gel immunodiffusion agar procedure was developed for detecting toxigenic strains of Clostridium botulinum type A. The method consisted of overlaying colonies grown on thin-layer tryptone-peptone-glucose-yeast extract agar with gel diffusion agar containing desired levels of C. botulinum type A antitoxin. Concentric precipitin zones formed around colonies of C. botulinum type A. Strains of C. botulinum type A were detected by this procedure. However, C. botulinum type B reacted to a lesser degree with this system. No reaction was noted with types E, F Langeland, F0, Clostridium perfringens, or with strains of nontoxigenic Clostridium sporogenes. Thickness of the plating medium, incubation time and temperature, environmental growth conditions, and levels of both agar and antitoxin were important factors affecting the efficiency of the procedure, whereas the age of the culture (used as inoculum) was not critical. Thin agar medium (5 ml per plate [15 by 100 mm]) containing 1.5% agar gave consistent results, but more agar limited diffusion, and lower levels encouraged spreaders. The optimal concentration of antitoxin incorporated into the gel diffusion agar overlay was 1.2 IU/ml gel diffusion agar. Rabbit type A antitoxin prepared with purer immunizing agent gave similar reactions. The addition of type A antitoxin in tryptone-peptone-glucose-yeast extract agar medium before inoculation with type A C. botulinum showed promising results.

Clostridium botulinum is distributed throughout the world and produces eight antigenically distinct neurotoxins (A through G). Types A, B, E, and F can cause human food-borne botulism, type C intoxicates birds, and type D intoxicates cattle (16). Various animal species have been known to be susceptible to type G (4). Types A, B, and recently, F were reported to cause infant botulism (1, 6). The neurotoxins of C. botulinum are produced within the cell and released by autolysis, degradation, or mechanical rupture of the cell wall (2). However, Siegel and Metzger (15) found that cell lysis may not be required to release the intracellular toxin.

The toxins of C. botulinum are routinely detected by a variety of techniques (3, 8–11, 13, 17, 18). Although the colonial morphology of the clostridia can be used as a guide, serological tests are still necessary to ascertain the type of C. botulinum (7, 16). At present, the assay of the toxin elaborated by C. botulinum is determined by mouse neutralization tests and by isolation of the bacteria with anaerobic culture technique. Therefore, in this communication we describe a fast in vitro procedure for identifying isolated colonies of C. botulinum type A with a simple immunodiffusion technique on an agar plating medium. Several factors that may affect the efficiency of this method were also examined.

MATERIALS AND METHODS

Microorganisms and media. Table 1 shows the origin and strains of all cultures used. C. botulinum type A strains 12 and 73A were utilized to develop the method, and all others were used for positive and negative confirmation. The organisms were maintained in cooked-meat medium and incubated at 35°C. The test organism was plated on tryptone-peptone-glucose-yeast extract agar (TPGYA) medium and incubated anaerobically in GasPak jars (BBL Microbiology Systems). The TPGYA medium consisted of (grams per liter of distilled water): glucose (50), tryptone (50), peptone (5), yeast extract (20), and Difco agar (10 to 15). All ingredients except glucose were dissolved by heating in distilled water, the pH was adjusted to the desired value with 1 N HCl or NaOH, and the mixture was then autoclaved for 15 min at 121°C. The glucose (50% aqueous solution) was filter sterilized with a 0.45-μm filter and then added to the tempered medium.

Preparation of spore crop. The spores were produced by inoculating cooked-meat medium with a 1%
TABLE 1. Sources of cultures used

<table>
<thead>
<tr>
<th>Culture</th>
<th>Strain</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type B</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Type C</td>
<td>62A, 73A</td>
</tr>
<tr>
<td></td>
<td>Type D</td>
<td>Green beans</td>
</tr>
<tr>
<td></td>
<td>Type E</td>
<td>Beluga</td>
</tr>
<tr>
<td></td>
<td>Type F</td>
<td>8G, Langeland</td>
</tr>
<tr>
<td>C. sporogenes</td>
<td>A</td>
<td>1979, A 2164, A 2673, A 2256, A 2133, A 1506, A 1507</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>11437</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>A</td>
<td>8797</td>
</tr>
<tr>
<td>Clostridium spp.</td>
<td>A</td>
<td>2222</td>
</tr>
<tr>
<td>Gram-positive, nonopore-forming anaerobe</td>
<td>A2672</td>
<td>C</td>
</tr>
</tbody>
</table>

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Gel-phosphate buffer. This buffer contained (grams per liter of distilled water): gelatin (2.0) and dihydrogen phosphate (4.0). The ingredients were dissolved with heat, the pH was adjusted to 6.2 with 1 N HCl or 1 N NaOH, and the mixture was autoclaved at 121°C for 20 min.

Preparation of TPGYA plates. Standard TPGYA plates contained 5 ml of medium in each sterile plastic petri dish (15 by 100 mm). The solidified medium was placed in the laminar flow hood to ensure surface drying. Test organisms were streaked with a laboratory inoculating needle or with a sterile glass rod and incubated in a GasPak anaerobic jar.

Identification of toxigenic C. botulinum. After incubation of TPGYA plates, GDA-antitoxin (50 to 55°C) was placed on all isolated colonies with a sterile pipette. The plates were then reincubated aerobically at the desired time and temperature and then flooded with thiazine red R stain for 10 to 15 min. The excess thiazine red R stain was poured off, and the plates were examined for zones of precipitation around colonies with indirect fluorescent lighting or incandescent light, using a dark background.

Toxin in liquid media and TPGYA. Toxicities of the cultures used were determined by the amount of toxin produced in cooked-meat medium or TPGYB (agar omitted from TPGYA). The medium was inoculated, incubated at 35°C (5 days for cooked-meat medium or 9 days for TPGYB), and centrifuged at 6,000 × g for 30 min at 5°C, and the supernatant was collected and adjusted to pH 6.2. Ten-fold serial dilutions were prepared in gel-phosphate buffer and injected into separate pairs of mice with 0.5 ml of the dilutions (12). The highest dilution which killed both injected mice within 3 days was considered the mouse lethal dose (MLD) per ml. C. botulinum type A antitoxin protected mice against challenges with twice the MLD of the culture broth. Two mice were also injected with the boiled, undiluted toxin broth and observed.

Isolated colonies of C. botulinum type A, cultures 73A and 12, were allowed to grow on TPGYA for 48 h. A 12-mm-diameter circle of agar beneath the surface of the colony was suspended in 2 ml of gel-phosphate buffer. The suspension was hand shaken to break up the agar and allowed to stand at room temperature for 1 h. The fluid was diluted with gel-phosphate buffer and assayed for toxin.

RESULTS AND DISCUSSION

Considerable growth of C. botulinum type A, strains 73A and 12, was observed on TPGYA medium over a wide range of pH (6.5 to 9.0), with a maximum for strain 73A occurring at pH 7.6 (Fig. 1). The latter pH also showed the maximum number of colony-forming units with a diameter of >1.0 mm. Figure 2 shows a standard TPGYA plate inoculated with C. botulinum strain 73A and overlaid on the right side only with GDA antitoxin. Two concentric precipitation zones surrounding each colony were clearly visible. All of the type A C. botulinum cultures gave similar positive results.
Fig. 1. Effect of pH of TPGYA media on growth and size of colony-forming units. A known volume of C. botulinum type A strain 73A organisms were spread over the plate surface and incubated anaerobically for 48 h at 35°C.

Fig. 2. Typical standard plate after TPGYA inoculated with C. botulinum strain 73A, incubated for 48 h at 35°C, and overlaid with the GDA-antitoxin for 24 h at 25°C (right side). Two definite, concentric precipitation zones surround each colony (right side).

Several factors affected the results. Table 2 summarizes the effect of different incubation times for the TPGYA and after making the GDA overlay on the diameter (millimeters) of the outer precipitation zone. A total of 48 h (24 h for TPGYA and 24 h for GDA-antitoxin) was the minimal incubation time needed to obtain precipitation zones. The precipitation zones did not increase either in size or intensity when inoculated TPGYA was incubated longer than 48 h, and reaction with GDA-antitoxin was more than 24 h. The TPGYA was incubated at 35°C, and the GDA-antitoxin was incubated at 25°C. One of the most critical factors was the volume of TPGYA; the best colonial growth and precipitation zones occurred when 5 ml of TPGYA medium per plate was used. Optimum agar concentration was 1.2 to 1.5%; less than 1.2% tended to promote development of spreaders, and greater than 1.5% limited diffusion of toxin.

The level of antitoxin in the GDA was another factor examined. Intensity of the precipitation zones decreased as the level of type A antitoxin was reduced from 1.2 to 0.3 IU/ml (Fig. 3).

The other cultures were tested by inoculating standard TPGYA, incubating for 48 h at 35°C for colony formation and overlaying with GDA containing 1.2 IU/ml, and incubated 24 h at 25°C. One small, faint zone was detected around type B colonies, whereas two more intense zones surrounded type A. The diameter of the inner zone for type A was similar to that of type B. It is believed that this cross-reaction may be due to the nontoxic hemagglutinin shared by C. botulinum types A and B (5, 9, 14, 17, 18). No precipitation zones were formed by the tested strains of C. botulinum types E or F, C. sporogenes, and C. perfringens (Fig. 4). This system was able to distinguish C. botulinum strain 73A from C. perfringens colonies developed by streaking a mixture of the two cultures, since zones of precipitation were not observed around the colonies of C. perfringens (Fig. 5).

No precipitation zones were noted around colonies of the 11 nontoxicigenic cultures composed of nine C. sporogenes (nontoxicigenic C. botulinum-like), one Clostridium sp., and one nonsporeforming, gram-positive rod.

Data obtained for the MLD/ml detected by

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>System used</th>
<th>Diam (mm) of precipitation zone of indicated culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>TPGYA</td>
<td>12 73A</td>
</tr>
<tr>
<td>24</td>
<td>GDA</td>
<td>4-7 4-7</td>
</tr>
<tr>
<td>24</td>
<td>TPGYA</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>GDA</td>
<td>6-8 6-8</td>
</tr>
<tr>
<td>48</td>
<td>TPGYA</td>
<td>10-12 10-12</td>
</tr>
<tr>
<td>48</td>
<td>GDA</td>
<td>10-12 10-12</td>
</tr>
</tbody>
</table>

*a No precipitation was observed after TPGYA incubation at 35°C.

*b Incubation at 25°C.
The type A antitoxin prepared with toxoid made of neurotoxin isolated from type A toxic crystals gave comparable results to that of the CDC antitoxin. Using either antitoxin preparation caused no zones of precipitation around colonies of C. sporogenes. When the former purified preparation was used, colonies of type A

Fig. 3. Effect of antitoxin level in GDA overlay on intensity of precipitation zones surrounding C. botulinum type A strain 73A. Three dilutions of antitoxin (1:5, 1:10, and 1:20, which correspond to 1.2, 0.6, and 0.3 IU/ml) were used.

The mouse bioassay procedure (7, 10) in the culture fluid for type A C. botulinum showed that the toxicity ranged from 2,000 to 200,000 MLD/ml; strain 51-317A had 2,000; Hall, 11A, 12, 62A, 69A, V-141, and 430A strains exhibited a level of 20,000; and 73A, 426A, and 429A strains had 200,000 MLD/ml. No direct relationship could be established between the levels of toxicity in culture fluid and the precipitation zone sizes, although the less toxic cultures sometimes gave smaller diameter or less intense precipitation zones or both. The agar beneath individual colonies of C. botulinum type A was found to be highly toxic. For strain 73A, an MLD of 40,000 was found in the agar associated with a single isolated colony, whereas for strain 12, the MLD was 4,000.

The intensities and sizes of the two concentric precipitation zones surrounding the individual colonies of C. botulinum type A strain 73A decreased in diameter with a change in concentration of glucose from 5.0 to 0.5% in TPGYA medium, whereas the size of the colony increased with the reduction in glucose concentration. We also examined inoculated TPGYA plates containing 1.2 IU/ml of the type A antitoxin. The latter was incorporated in TPGYA medium (55°C) before pouring into plates. Two concentric zones surrounding the colonies of C. botulinum type A were observed after 48 h of incubation at 35°C, but growth was inhibited, apparently by the antitoxin in the medium.

Fig. 4. Interaction of type A antitoxin in the GDA (1.2 IU/ml) overlay with colonies of types A12, A60, A7s, E, F60, F7 (Langeland), C. perfringens (Cp) and C. sporogenes (Cs) on standard TPGYA plate.

Fig. 5. Typical standard TPGYA plate inoculated with C. botulinum strain 73A and C. perfringens mixture. Isolated colonies of C. perfringens and C. botulinum were overlaid with GDA containing 1.2 IU of type A antitoxin per ml of GDA.
C. botulinum had a single zone of precipitation, in contrast with the double zone obtained with CDC antitoxin. Naturally, the best antitoxin is produced by using the purest toxin available for animal inoculation. The CDC antitoxin was chosen since it was readily available and gave consistent results which were satisfactory for this procedure. The advantages of this method are as follows: (i) individual toxic colonies of C. botulinum type A can be selected from nontoxic organisms; (ii) it is relatively rapid and simple; and (iii) other methods are not able to identify individual colonies of C. botulinum which must be tested further to prove their purity and toxicity. We do not regard the ability of the type A antitoxin used in the procedure to detect type B C. botulinum organisms as a real disadvantage. The appearance of the type B precipitate zone is easily differentiated from that of type A. The most important factor is the presence of C. botulinum; yet, type A can be distinguished from type B on the plate.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Craig Smith, Food Science Department, University of Georgia, Athens. We also thank C. Hatheway of the Centers for Disease Control for his technical help and H. Sugiyama for his suggestions and for supplying the type A C. botulinum antitoxin used for confirmation studies.

LITERATURE CITED