An ELISA for Detection of Botulinal Toxin Types A, B, and E in Inoculated Food Samples

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed to screen for the presence of botulinal toxin types A, B, and E in inoculated food studies. A commercially available trivalent antitoxin (Connaught Laboratories, Ontario) was used as a capture antibody and biotinylated for use as a secondary antibody. An avidin-alkaline phosphatase conjugate coupled with an enzyme-antitoxin (Connaught Laboratories, Ontario) was used as a capture for types B and E, respectively. Toxin produced by two-type F strains (proteolytic and nonproteolytic) was detected in a liquid laboratory medium. In a comparative study of over 490 samples of ground turkey meat inoculated with C. botulinum types E and nonproteolytic B, the ELISA gave no false negatives and 91 false positives. False positives were thought to be due to the presence of inactivated toxin or toxin levels insufficient to cause mouse death. Statistical analysis of these data showed an ELISA sensitivity of 100%, specificity of 70.6%, and an efficiency of 81.4% when compared to the mouse bioassay for detection of botulinal toxins types B and E. Coffee intermediates inoculated with proteolytic Clostridium botulinum types A and B caused nonspecific death in mice but were negative for presence of toxin by ELISA.

Clostridium botulinum, a ubiquitous organism found in both terrestrial and aquatic environments, produces an extremely potent neuroparalytic toxin. Although outbreaks of foodborne botulism are rare, consumption of foods containing botulinal toxin causes severe illness or death. Foods historically associated with foodborne botulism are low-acid canned foods, especially meats and vegetables, and meats or fish preserved by smoking, salting, or curing (6). C. botulinum is also a concern in other products, i.e., those which do not conform to federal regulation for low-acid canned foods such as pasteurized processed cheese spreads (10).

Current trends in marketing include extended shelf-life refrigerated convenience foods which may receive little or no heat prior to consumption. This, in combination with consumer demand for “fresh” food, poses a special challenge for food processors. Several strains of C. botulinum implicated in human botulism outbreaks are capable of growth at or slightly above refrigeration temperatures (1,17). Minimum recorded growth temperatures for Group I (type A and proteolytic strains of types B and F) and Group II (type E and nonproteolytic strains of types B and F) are 10 and 3.3°C, respectively (9,14).

In the absence of preservatives, other barriers such as water activity, salt, reduced pH, and natural antimicrobials may be present or incorporated into product formulations to act in combination with low temperatures to delay or prevent growth of C. botulinum and subsequent toxigenesis (7). Efficacy of these barriers must be determined not only for shelf-life assessment of various product formulations but may also be required in the future to assure regulatory agencies of product safety.

The accepted and most sensitive assay for C. botulinum toxin is the in vivo mouse bioassay (19,21). Limitations are that the method is slow, taking up to 4 d for completion, and it cannot be used if samples contain other lethal substances which may cause nonspecific deaths. In an inoculated food study designed to determine under what parameters a product would be formed in a product, detection of only biologically active toxin by the mouse bioassay can be an additional limitation. Were toxin produced in a food sample to be rendered inactive, i.e., through endogenous enzymes in the food, it would no longer be evident using the mouse bioassay, thereby giving a false impression as to the safety of a product under the conditions of that variable.

Neutralization tests for typing and quantitative determination of toxicity require large numbers of mice, and animal facilities are costly to maintain. They are also labor intensive, politically sensitive with regards to animal rights, and not available to most food microbiology laboratories. Therefore, toxin detection and, consequently, safety assessment of products could be better facilitated by a rapid, sensitive, and specific in vitro assay for botulinal toxins.

One of the most sensitive in vitro methods is the enzyme-linked immunosorbent assay (ELISA) (18,23). ELISA procedures detecting 10 to 50 minimal lethal dose (MLD) of botulinal toxin type A, 20 MLD type B, and 80 MLD type E have been described in the literature.
(2,5,7,18,20). Most of these assays utilize monoclonal capture antibodies and signal amplification to increase specificity and sensitivity.

The intent of this study was to develop an ELISA suitable for large-scale screening of food samples for botulinal toxins such as would be encountered in challenge or predictive modeling studies. Challenge testing of foods with *C. botulinum* usually involves inoculation of foods with several different strains of types A, B, and E depending on product type, process given, storage conditions, etc. After incubation, foods are assayed for the presence or absence of botulinal toxin. In the present study rather than using three separate assays to detect botulinal toxins of types A, B, and E, a combination of antibodies for these toxins was used in a single assay for detection of toxic samples. The resultant trivalent botox ELISA was then assessed for sensitivity and specificity for botulinal toxins types A, B, E, and F in model systems and types B and E in foods.

**MATERIALS AND METHODS**

**Toxins, culture strains, and antibodies**

Purified botulinal neurotoxins types A, B, and E were obtained from Dr. Bibhuti DasGupta, Food Research Institute, University of Wisconsin-Madison. Toxins were held in an ammonium sulfate suspension to avoid decay, and small amounts were held in sodium phosphate buffer (pH 7.5) for use as working stock solutions. A description of strains in the study and their sources are listed in Table 1.

Trivalent botulism antitoxin - types A, B, and E Lot #3003, Connaught Laboratories, Toronto, Ontario, Canada, was provided by the SIDS/Infant Botulism Laboratory, State of California, UC Davis Laboratory. The polyclonal antitoxin, which is currently commercially available, is a pool of the immunoglobulin G fraction of three separate antisera produced in horses. Composition of the antitoxin in World Health Organization Units is 1049, 738, and 3082 units per ml for types A, B, and E, respectively.

**Test buffers and diluents**

Carbonate coating buffer, pH 9.6, contained (g/L glass-distilled water): Na₂CO₃, 1.59; NaHCO₃, 2.93. Tris-buffered saline with 0.05% Tween 20, pH 7.5 (TBST), contained (g/L glass-distilled water): Tris-HCl, 7.9; NaCl, 11.7; KCl, 0.23; Tween 20, 0.5; pH was adjusted with 10 N NaOH. Phosphate-buffered saline with 0.05% Tween and 0.5% casein (PC buffer) contained (g/L glass-distilled water): KCl, 0.2; NaCl, 8.0; KH₂PO₄, 0.2; Na₂HPO₄, 1.25; Tween 20, 0.5; 5% casein solution, 100 ml. Casein solution (5%) contained 50 g casein in 800 ml 0.3 M KOH; after stirring overnight the pH was adjusted with 5 N HCl and volume adjusted to 1000 ml with glass-distilled water. Gelatin phosphate buffer, pH 6.2 (GPB), contained (g/L distilled water): gelatin, 2; Na₂HPO₄, 4. Phosphate buffer, pH 6.0, was prepared by adding a 0.2 M solution of Na₂HPO₄ to a 0.2 M solution of NaH₂PO₄ until pH 6.0 was attained.

**Biotinylation of Connaught trivalent antibody (CTA)**

Connaught trivalent antitoxin (types A, B, and E) was biotinylated in the following manner: Antibody concentration was adjusted to 1.5 mg/ml and dialyzed overnight at 4°C against 0.1M NaHCO₃, pH 8.6, and then adjusted to 1.2 mg/ml bicarbonate buffer by A₂₈₀. Seventy-eight microliters of a 2 mg/ml solution of biotinamidocaproyl-N-hydroxysuccinimide (Sigma B2643) in dimethyl sulfoxide was added dropwise while stirring. The antibody was again dialyzed overnight against phosphate-buffered saline and stored at 2°C.

**TABLE 1. Source of cultures used in this study.**

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<th>Species</th>
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* 1, Culture collection, C. Genigeorgis, Davis, CA; 2, W. H. Lee, FSIS, USDA, Beltsville, MD; 3, Food Science Dept., University of California, Davis, CA; 4, American Type Culture Collection, 5, State of California Infant Botulism Laboratory, Davis, CA.

**ELISA amplification**

In brief, amplification is based on enzyme catalyzed, cyclic oxidation/reduction of NADH with concurrent reduction of iodonitrotetrazolium dye to a colored end-product, formazan; for each molecule of original substrate reduced, hundreds of formazan molecules can be generated.

**Trivalent botox ELISA procedure**

Flat-bottom microtiter plates (NUNC-Immuno Maxisorp Plates, NUNC, Inc., Naperville, IL) were coated with 100 μl of a 1/1,000 dilution of Connaught trivalent botulism antitoxin types A, B, and E (CTA) in carbonate coating buffer, pH 9.6. After overnight incubation at 4°C, wells were washed five times with Tris-buffered saline with 0.05% Tween 20 (TBST), pH 7.5, with a 45 s delay between washings.

Non-specific binding sites were blocked with 200 μl of 1% fetal calf serum (GIBCO, heat inactivated) in phosphate-buffered saline with 0.05% Tween 20 and 0.5% casein (PC buffer), pH 7.4, and incubated 90 min at 37°C. After washing as described above, duplicate wells each received 100 μl of standard neurotoxin solution or test sample. Negative control wells (8 per plate) received 100 μl of Tris-buffered saline with 0.5% Tween 20. Plates were incubated 60 min at 37°C. Plates were washed and 100 μl of a 1/1,000 dilution of biotinylated CTA in PC buffer was
added to each well. Plates were incubated for 60 min at 37°C and washed.

ExtraAvidin alkaline phosphatase (Sigma, St. Louis, Mo.) was diluted 1/6,000 in PC buffer and 100 μl pipetted into each well. Plates were incubated at 37°C for 60 min and washed five times with TBST. The fifth wash was left for 10 min, and plates were emptied and pounded dry on paper toweling to remove any traces of free phosphates. The ELISA Amplification System was used according to the manufacturer’s instructions except that plates were incubated at 37°C for 25 min after addition of substrate and amplifier. Fifty microliters of stop solution (0.3 M H₂SO₄) was then added per well. A₄₉₀ was determined immediately after addition of stop solution.

Production of spore crops

Spore crops were produced in a liquid sporulation medium and cleaned by centrifugation in a density gradient using the method of Kihm et al. (11). Flasks of sporulation medium were inoculated from 24-h cultures and incubated until high levels of refractile spores were observed in the spore cell layer which had accumulated in the bottom of the flask (up to 5-8 d depending upon the strain).

Contents of the flask were centrifuged, and the resultant pellet was washed 10 times with sterile double-distilled water and resuspended in 20 ml of sterile double-distilled water. Five milliliters of the spore suspension was then layered onto a 62% aqueous solution of diatrizoate meglumine (Renograin 60, Bristol Meyers/Squibb) and centrifuged at 18,800 x g for 20 min. Cells and debris which remained in the upper layer were decanted off, and the pellet of free refractile spores was washed 10 times in sterile double-distilled water and stored at 4°C.

Trypsin activation of test samples

All test samples inoculated with C. botulinum type E and nonproteolytic B, i.e., broth culture supernatants, food extracts, etc., were trypsin activated using a modification of the Center for Disease Control protocol. Sample extract (0.2 ml), 0.3 ml gelatin phosphate buffer (pH 6.2), and 0.1 ml of a 1% trypsin (Difco, 1:250) solution were mixed in a tube and incubated at 37°C for 30 min. The trypsinized extract was used within 30 min in the mouse bioassay. Mouse bioassay for toxin detection was done according to procedures outlined in the Food and Drug Administration Bacteriological Analytical Manual (22).

Trypsin activation of purified botulism neurotoxin type E

Purified botulism neurotoxin type E was trypsin activated by centrifuging 50 µl of an ammonium sulfate suspension of botulism neurotoxin type E in a 250-µl microcentrifuge tube at 10,000 x g for 5 min. Supernatant was drawn off and 1 mg of trypsin (Sigma # T-8642) in 50 µl 0.02M phosphate buffer (pH 6.2) and 0.1 ml of a 1% trypsin (Difco, 1:250) solution were mixed in a tube and incubated at 37°C for 30 min. The trypsinized extract was used within 30 min in the mouse bioassay. A mouse bioassay for toxin detection was done according to procedures outlined in the Food and Drug Administration Bacteriological Analytical Manual (22).

Generation of standard curves for purified neurotoxins in buffer

Standard curves for each neurotoxin in a buffer solution were generated in the following manner. Purified neurotoxin solutions obtained from Dr. DasGupta were diluted in gelatin-phosphate buffer pH 6.2 (GPB). Serial 1:2 dilutions were prepared in GPB to give a range of concentrations from 10 pg/ml to 10,000 pg/ml. Picogram concentrations were approximate and derived from protein concentrations of the neurotoxin solutions as provided by Dr. DasGupta. Duplicate samples from each dilution were run in the ELISA and also injected intraperitoneally (i.p.) into mice in order to assess the sensitivity of the ELISA. The average of the two ELISA readings were plotted.

Comparison of toxin detection by ELISA and mouse bioassay in fresh broth culture supernatants

Supernatants of fresh broth cultures were tested in duplicate in the mouse bioassay and the ELISA to compare sensitivities of the two methods. Broth cultures of C. botulinum strains (proteolytic - A 62, B OKRA, F FT 42 and nonproteolytic - B 17, E Beluga, F 187) were prepared by inoculating heat-shocked spores (proteolytic strains 80°C/10 min, nonproteolytic strains 60°C/30 min) into freshly prepared brain heart infusion (BHI) with 0.1% cysteine and incubating at 37°C for 72 h in anaerobic chamber. Cultures were then centrifuged at 18,800 x g for 10 min and supernatants used for toxicity testing in mice and ELISA assays. Serial tenfold dilutions of the supernatants were made in GPB (pH 6.2). Only that portion of supernatant from nonproteolytic culture used for mouse i.p. injection was trypsin treated; ELISA testing was done on nontrypsin-treated sample.

Determination of specificity of ELISA for botulinal toxins A, B, and E in broth culture supernatants

In order to determine specificity of the ELISA, fresh broth culture supernatants of several strains of Clostridium sp. (botulinual and nonbotulinual) were surveyed for reactivity. Cultures were grown as described above, except that undiluted 48-h culture supernatants were used rather than 72-h cultures.

Detection of botulinal toxin from inoculated ground turkey meat samples using ELISA and mouse bioassay

A multifactorial study of time-to-toxigenesis for ground turkey meat samples inoculated with nonproteolytic C. botulinum spores was done using the mouse bioassay for toxin detection (16). Ground turkey meat samples were used to compare methods of detection. Strains used in the study were B 2, B 17, B 194, B 706, E 211, E 250, E KA 2, and E Beluga. Gelatin-phosphate buffer extracts of samples tested for botulinal toxicity by the mouse bioassay were randomly selected and tested for toxin in the ELISA; in total 490 samples were tested by the ELISA for presence or absence of botulinal toxin.

RESULTS AND DISCUSSION

Standard curves for purified neurotoxins in buffer

Standard curves for botulinal toxins types A, B, and E are shown in Fig. 1. LD₅₀ (i.p. injection) as determined by titration of ammonium sulfate suspensions of purified neurotoxins in mice for toxin types A, B, and E were 5 pg, 23 pg, and 37 pg, respectively, as noted in Fig. 1. The threshold for a positive ELISA reading (A₄₉₀ 0.116) was determined by calculating the mean plus two times the standard deviation of 12 control blanks as described by Feldkamp and Smith (4). Comparison of intraperitoneal LD₅₀ in mice to ELISA standard curves show that the limit of detection of the ELISA was nine LD₅₀ for toxin type A and less than one LD₅₀ for toxin types B and E, respectively.

In the original determination of LD₅₀ in mice for type E toxin, neurotoxin was diluted to 10,000 pg/ml prior to trypsin treatment. ELISA readings were similar to those shown in Fig. 1 for type E toxin, but no dilution caused death in mice, including the lowest dilutions containing 10,000 pg/ml. In a second experiment a small quantity (50 µl) of neurotoxin was trypsin treated, the reaction stopped with trypsin-chymotrypsin inhibitor, and then further dilu-
There are several possibilities for these apparent false-positive reactions. Biologically inactive toxin may be present, i.e., toxin not yet activated by proteolytic enzymes (as was seen in the first method of trypsin treatment of purified neurotoxin E) or overdigestion of toxin by i) proteolytic enzymes during trypsinization of the nonproteolytic strain (B 17) or ii) endogenous enzymes in the case of the proteolytic strain (F FT 42). Biologically inactive toxin can also compete with biologically active toxin for receptor sites in the mouse and consequently reduce its effectiveness in the biological system. Since the ELISA can detect both active and inactive toxin, the false positives may be due to detection of toxin levels which have insufficient activity to cause mouse death.

Another phenomenon being investigated is the possibility that the capture/signal antibody may be reacting to the nontoxic hemagglutinin molecule produced by the cell along with the toxin. The hemagglutinin molecule is much larger than the toxin molecule and could be detected before toxin levels are high enough to produce biological toxicity.

Capture or signal antibody may also be reacting to a somatic antigen or other protein produced by the organism. If so, it may be possible to remove this reactivity from the antibody by adsorption with that antigen or protein.

An area needing further investigation is the variance in A₄₉₀ observed for media control blanks. The ELISA test samples were distributed over two plates with eight wells on each plate used for media control blanks. On one plate A₄₉₀ of the controls ranged from 0.018 to 0.069 with a standard deviation of 0.017. The second plate had a much higher range (0.005-0.333) with three of the readings being over 0.1 and a standard deviation of 0.107. However, all inoculated broth dilutions which were positive for toxin by mouse bioassay had A₄₉₀ readings above the media control blanks.

**Determination of ELISA specificity for botulinal toxins A, B, and E, in broth culture supernatants**

Results of ELISA testing of broth culture supernatants of clostridial strains are given in Fig. 3. It should be noted that broth cultures used for this experiment were incubated 48 h as compared to 72 h incubation for broth cultures used in Fig. 2. Of the botulinal strains tested, only those strains producing types C and G toxin did not give positive A₄₉₀ readings. Although the antitoxins present in the Connaught trivalent antitoxin are for toxin types A, B, and E, reaction with toxin types F and D and slight reaction with Clostridium tetani may be due to homology between segments of their toxins and segments of type A, B, or E toxins. (3,12). The broth culture supernatant of Clostridium butyricum ATCC 41381 which produces type E botulinal toxin gave a positive ELISA response; Clostridium barati ATCC 43755 which produces type F botulinal toxin did not.

Reaction of broth culture supernatants of PA 3679 and Clostridium sporogenes ATCC 11437 in the ELISA needs further investigation, and experimentation is currently in progress to determine whether this phenomenon will occur in food systems or in 1:10 dilutions of similar broth cultures. One possible explanation is that a somatic antigen common to both C. botulinum and C. sporogenes is react-
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Figure 2. Comparison of Trivalent Botox ELISA to mouse bioassay for detection of botulinal toxins in serially diluted 48-h broth culture supernatants (BHI with 0.1% cysteine). ■ = positive mouse bioassay, □ = negative mouse bioassay. ----- media blank for ELISA. ELISA A490 greater than media blank considered positive.


Detection of botulinal toxin in inoculated turkey samples by ELISA and mouse bioassay

The threshold for a positive ELISA reading (A490 0.188) was determined by calculating the mean of uninoculated turkey controls plus 2x standard deviation as described by Feldkamp and Smith (4). Out of 490 total samples tested in both ELISA and mouse bioassay, 180 were positive and 219 were negative by both assays; ELISA results gave no false negatives and 91 false positives. Statistical analysis of these data using the method of Martin et al. (14) shows an ELISA sensitivity of 100% and specificity of 70.6% when

ing with the antibody since these strains have almost 100% DNA homology (13).

In screening of inoculated samples, spore levels of naturally occurring C. sporogenes may be substantially lower than those of C. botulinum inoculated into the product. Greenberg et al. found the mean level of putrefactive anaerobic sporeformers to be 2.8/g in a survey of 2,358 samples of pork, beef, and chicken (8). Therefore, if this is truly a cross-reaction to C. sporogenes in the ELISA, it should not be at levels high enough to cause false positives in an inoculated pack study.
compared to the mouse bioassay for detection of botulinal toxin. The overall efficiency of the ELISA (probability of a given result being correct) was 81.4%.

All samples showing toxicity by the mouse test were determined as positive in the ELISA visually without the aid of an ELISA reader \((A_{490} \geq 0.824)\). Of the 91 false-positive ELISA results, 30 samples were strongly positive \((A_{490} \geq 0.824)\), and the remainder had \(A_{490} > 0.188\) (threshold for positive ELISA) and < 0.824. As mentioned previously, these false positives may be due to detection of biologically inactive toxin, hemagglutinin molecules, other somatic antigens, or toxin levels lower than are detectable by the mouse bioassay. This is an area needing further investigation in order to draw a more definitive conclusion.

Preliminary studies have also been done with minced beef and fish which were processed under sous vide conditions and then inoculated with proteolytic and nonproteolytic strains of \(C.\) botulinum, types A, B, E, and F. Sixty-three beef and fish samples were tested for presence or absence of botulinal toxin by the mouse bioassay and the ELISA. Results were similar to those of the turkey study. No false negatives were detected by the ELISA, while there was a percentage of false positives as compared to the mouse bioassay. Of the 63 samples tested, 24 were negative by both assays, 9 were positive by both assays, 30 gave positive ELISA readings but did not cause mouse death, and no sample having a negative ELISA reading caused mouse death.

In another preliminary study, frozen samples of concentrated coffee syrups had been inoculated with proteolytic \(C.\) botulinum types A and B. Samples previously tested by mouse bioassay for botulinal toxins were then tested in the ELISA. Two samples containing 45% coffee solids caused nonspecific deaths in the mouse bioassay, probably due to caffeine concentrations above the \(LD_{50}\) for caffeine in mice of 127 mg/kg (oral \(LD_{50}\)). Samples contained approximately 500 mg caffeine per kg mouse. These samples were shown to be negative in the ELISA.

The cost of this ELISA is less than the mouse bioassay (approximately 10 U.S. dollars for a 96-well plate which can contain 30-40 samples, excluding labor). Considering the fact that no false negatives have been detected to date and given the current attitude towards animal testing, this ELISA may prove to be a useful tool in screening samples for presence of botulinal toxins in inoculated pack studies.

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