APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2006, p. 1231–1238 Vol. 72, No. 2
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.2.1231–1238.2006

Detection of Type A, B, E, and F *Clostridium botulinum* Neurotoxins in Foods by Using an Amplified Enzyme-Linked Immunosorbent Assay with Digoxigenin-Labeled Antibodies

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Received 30 June 2005/Accepted 29 November 2005

An amplified enzyme-linked immunosorbent assay (ELISA) for the detection of *Clostridium botulinum* complex neurotoxins was evaluated for its ability to detect these toxins in food. The assay was found to be suitable for detecting type A, B, E, and F botulinum neurotoxins in a variety of food matrices representing liquids, solid, and semisolid food. Specific foods included broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meat products, and dairy foods. The detection sensitivity of the test for these botulinum complex serotypes was found to be 60 pg/ml (1.9 50% lethal dose [LD_{50}]) for botulinum neurotoxin type A (BoNT/A), 176 pg/ml (1.58 LD_{50}) for BoNT/B, 163 pg/ml for BoNT/E (4.5 LD_{50}), and 117 pg/ml for BoNT/F (less than 1 LD_{50}) in casein buffer. The test could also readily detect 2 ng/ml of neurotoxins type A, B, E, and F in a variety of food samples. For specificity studies, the assay was also used to test a large panel of type A *C. botulinum*, a smaller panel of proteolytic and nonproteolytic type B, E, and F neurotoxin-producing *Clostridia*, and nontoxigenic organisms using an overnight incubation of toxin production medium. The assay appears to be an effective tool for large-scale screening of the food supply in the event of a botulinum neurotoxin contamination event.

Clostridium botulinum is an anaerobic, gram-positive, spore-forming rod that produces a potent neurotoxin. There are seven types of botulinum toxin designated by the letters A through G. Types A, B, and E are most commonly associated with illness in humans. Type F is implicated less often, and types C and D are rarely associated with human botulism. Type G has never been linked to human botulism. Botulinum neurotoxins (A to G) are large proteins; each has antigenically distinct properties but shares the pharmacological characteristics that cause the flaccid muscle paralysis that characterizes the disease botulism (39). A botulinum neurotoxin (BoNT) complex is formed by the synthesis of a botulinum neurotoxin and, depending on the serotype, two to six nontoxic neurotoxin associated proteins (NAPs). NAPs are known to protect the BoNTs from the acidic environment and proteases of the gastrointestinal tract (33, 37, 38, 41). This protective complex is partly responsible for making BoNTs the most potent natural food poisoning agents known (26, 29, 40).

Preventive measures for deliberate botulinum toxin food contamination are a growing concern. Much effort has also been expended by the food industry to ensure that food treatment processes prevent the growth and toxin production of *C. botulinum*. It is not only essential to detect the botulinum toxin contamination in a particular food supply but also critical that an early and accurate determination of serotype be made so that the appropriate antitoxin for the disease can be administered. Therefore, there is a need for rapid, sensitive, and specific assays that can detect and differentiate the toxin type. Currently, the only accepted method for the detection of botulinum toxin in foods is the mouse bioassay. In this assay, food extracts are prepared and then injected intraperitoneally into mice. The animals are observed for botulism symptoms for up to 48 h (17).

Although the method is sensitive and detects type A botulinum complex toxin down to 10 pg/ml, the number of samples that can be assayed is severely restricted, and it can take up to 2 days for positive samples to be identified as containing botulinal toxin. A neutralization assay is needed to identify the toxin type. Alternatively, rapid in vitro methods, such as the enzyme-linked immunosorbent assay (ELISA), require only 5 to 6 h for detection and toxin type identification of BoNTs. Several laboratories have developed ELISA detection assays designed for sensitivity approaching that of the mouse bioassay (10–12, 14, 17–20, 31, 32, 44, 48, 49). While these detection systems mostly employ differences in enzyme substrates and sources of antibodies, the basic principle of immunodetection is the same.

Doellgast et al. (12) reported an enzyme-linked coagulation assay with a toxin detection sensitivity comparable to the mouse bioassay. This sensitive method relied on a sophisticated amplification system utilizing a snake venom coagulation factor but was limited by its complexity and reagent expense (31). The enzyme-linked coagulation assay combinations of antibodies (chicken antibodies and horse biotinylated antibodies) have a major limitation in their application for food testing. For example, the chicken antibodies cannot be used to test samples in which either chicken meat or egg yolk are present, and the biotinylated antibodies cannot be used in samples containing either biotin or avidin (e.g., egg white and milk) (12).

Recently, we developed a sensitive and specific ELISA to

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detect *C. botulinum* neurotoxins A, B, E, and F. The assay uses toxin type-specific polyclonal antibodies to capture the toxin and digoxigenin (DIG)-labeled toxin type-specific polyclonal antibodies as secondary antibodies. These DIG-labeled secondary antibodies are then detected by anti-DIG antibody conjugated to horseradish peroxidase. This enzyme is then detected using a chromogenic substrate. In this work, we demonstrate the application of the amplified ELISA for the detection of BoNTs in a variety of different food samples and for the detection of botulinum toxin in botulinum strain culture.

MATERIALS AND METHODS

Pure type A, B, E, and F neurotoxin complex toxins were purchased from Metabiologics, Inc. (Madison, WI). These complex toxins were derived from type A Hall strain, type B Okra strain, type E Alaska strain, and type F Langeland strain. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the toxin neurotoxin complex indicated a pure neurotoxin complex preparation. Their size and toxicity are described on the certificates obtained from Metabiologics, Inc., and are as follows, respectively: type A neurotoxin complex, 500 kDa and 3.2×10^7 50% lethal dose [LD_{50}]/mg; type B neurotoxin complex, 550 kDa and 9.0×10^6 LD_{50}/mg; type E neurotoxin complex, 300 kDa and 2.8×10^7 LD_{50}/mg (trypsin activated); and type F neurotoxin complex, 280 kDa and 2.2×10^6 LD_{50}/mg. Liquid, solid, and semisolid food samples were purchased from local grocery stores. Fresh raw milk was obtained from the U.S. Department of Agriculture, Agriculture Research Service (Beltsville, MD). Ice cream was stored at −20°C while other food samples were stored at 4°C until used in the experiment. Bovine serum albumin was purchased from Sigma Chemical Co. (St. Louis, MO). The protein concentration was measured at 595 nm on an ELx 801 Ultra Micro plate ELISA plate reader (Bio-Tek Instrument, Winooski, VT) using a microtiter plate protocol. The protein concentration was determined using a Bio-Rad (Hercules, CA) protein assay kit and the procedure of Bradford (4).

Affinity purification of capture anti-BoNT antibodies. Antibodies against A, B, E, and F serotypes were purified from hyperimmune goat, rabbit, or horse serum. A HiTrap Protein G HP Column (Amersham Biosciences, Piscataway, NJ) was prepared by following the manufacturer's instructions. The serum (1 ml) was centrifuged at 10,000 × *g* at 4°C in a centrifuge tube to remove any particulate matter. The clarified supernatant was removed by a Pasteur pipette and diluted 1:10 in 0.01 M phosphate-buffered saline (PBS), pH 7.2. The column was then equilibrated with 0.01 M PBS, pH 7.2, at a flow rate of 4 ml/min. A 10-ml volume of the diluted serum was then applied to the column at a flow rate of 4 ml/min. The column was then washed with 0.01 M PBS, pH 7.2, until the absorbance of the wash buffer was recorded as <0.2 at 280 nm. This was achieved by using approximately 10 column volumes of PBS. The immunoglobulin G (IgG) was eluted with 0.1 M glycine buffer, pH 2.7. Three-milliliter volume fractions were collected in glass tubes containing 300 μl of 1 M Tris-HCl, pH 9.0. The fractions were collected until the absorbance of the last fraction was <0.1 at 280 nm. The fractions were pooled, and the protein concentration was determined by the Bradford assay. The IgG was then precipitated using 100% saturated ammonium sulfate to obtain a final concentration of 50% ammonium sulfate, with storage overnight at 4°C. The precipitated IgG was recovered by centrifugation at 4°C and 10,000 × *g* for 30 min. The IgG pellet was dissolved in PBS or in a 0.1 M bicarbonate buffer, pH 8.0, before being used for the experiments. The protein concentration was determined using an extension coefficient of 1.3 M^{-1} at 280 nm (23, 47).

DIG labeling of affinity-purified antibody. A Roche DIG-protein labeling kit (item 1 367 200) was used to label the botulinum IgGs. The digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy succinimide ester (DIG-NHS; Roche Diagnostics, Mannheim, Germany) solution was freshly prepared before labeling. To prepare a working solution of DIG-NHS, a 50-μl volume of dimethyl sulfoxide was added to the reaction vial containing DIG-NHS. The vial was vortexed vigorously several times to dissolve the DIG-NHS. This generated a 20 mg/ml solution of DIG-NHS in dimethyl sulfoxide. Our procedure deviated from the manufacturer's recommended buffer (PBS supplied with the DIG protein labeling kit). The best results were obtained using 0.1 M carbonate buffer, pH 8.0 to 8.4, instead of PBS. The IgGs were dialyzed in 0.1 M sodium carbonate buffer, pH 8.4, prior to DIG labeling. One milligram of IgG to each of the neurotoxins A, B, E, and F was individually prepared in 1 ml of carbonate buffer. The DIG-NHS was used in a 1:50 molar ratio (IgG/DIG-NHS) by adding 11.7 μl of the DIG-NHS solution to 1 ml of IgG (1 mg/ml) and incubating for 2 h at room temperature (25°C) with gentle stirring. The nonreacted remaining DIG-NHS was separated by gel filtration on a Sephadex G-25 column. The reaction mixture was applied to the column, and this step was followed by a 5-ml wash with carbonate buffer. The first three 1-ml fractions containing labeled protein were collected, pooled, and stored at 4°C. The protein concentration was determined using an extinction coefficient of 1.3 M^{-1} at 280 nm.

Cultures and growth of *C. botulinum* strains. A total of 77 strains of type A and 12 strains each of types B, E, and F, including both proteolytic and nonproteolytic type B and type F strains (isolated from different environmental and clinical sources), were grown anaerobically in a Bacto-cooked meat medium (Difco Laboratories, Detroit, MI) for 16 h. Strains of proteolytic types A, B, and F were grown at 37°C, while nonproteolytic types B, E, and F were grown at 30°C. One hundred microliters of the overnight cultures grown from stock cultures were streaked on a trypticase-peptone-glucose-yeast extract agar plate and incubated anaerobically for 24 h. The agar plates were incubated at 37°C for proteolytic types A, B, and F and at 30°C for nonproteolytic types B, E, and F. A single colony from each plate was then transferred into 10 ml of trypticase-peptone-glucose-yeast extract broth, and the medium was incubated at 35°C for proteolytic types A, B, and F and at 30°C for nonproteolytic types B, E, and F for 24 h. The culture material was clarified by centrifugation at 10,000 × *g* for 10 min, followed by sterile filtration through a 0.45-μm-pore-size bacteriological Acrodisc filter. Culture filtrates were stored at 4°C until used.

Sample preparation. Control samples of botulinum neurotoxin complex A, B, E, or F (range, 0.2 to 10 ng/ml) were prepared in 500 μl of casein buffer (1% casein in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4). The pH of liquid food samples was adjusted to 7.2 before samples were spiked with the neurotoxin complex. The toxin concentration was adjusted to produce a final concentration of 2 ng/ml prior to the ELISA. Five-milliliter liquid food samples of orange juice, bottled water, soft drinks, vanilla extract, and apple juice were spiked with toxin to yield 4 ng/ml of pure neurotoxin complex A, B, E, or F, mixed by shaking, and then incubated for 30 min at room temperature. The samples were then centrifuged at 7,000 × *g* (Eppendorf Brinkmann, Inc., N.Y.) for 30 min at 4°C to remove solid particles. Subsequently, the supernatant was diluted 1:1 with the casein buffer in a glass test tube, thoroughly mixed, and used for the assay.

High fat content and viscous foods such as ice cream, milk, and honey were spiked with 10 ng of pure botulinum neurotoxin complexes A, B, E, and F per ml of food, mixed by shaking, and incubated for 30 min at room temperature. The samples were then diluted 1:5 with the casein buffer in a glass tube and mixed thoroughly with gentle vortexing. The diluted mixture was then centrifuged at 7,000 × *g* for 30 min at 4°C to remove the lipid layer. The aqueous supernatant was carefully removed and used for the assay.

For semisolid foods, a 10-g sample of food was spiked with 20 ng of each botulinum neurotoxin complex A, B, E, and F in separate wide-mouth vials. The toxin complex was introduced uniformly over the surface of the food samples, and samples were then incubated for 30 min at room temperature. After incubation, the samples were mixed with 10 ml of sample buffer and homogenized with a benchtop stomacher (Seward, Cincinnati, OH) to make a homogeneous suspension. For solid foods, a 10-g portion of the food sample was spiked with 20 ng of pure neurotoxin complex A, B, E, or F in wide-mouth glass vials. The toxin complex was introduced uniformly over the surface of the food samples, and the samples were then incubated at room temperature for 30 min. The food portion was then chopped in to small pieces by hand, thoroughly mixed with 10 ml of sample buffer, and homogenized with a benchtop stomacher to make a homogeneous suspension. The food-buffer mixtures of both semisolid and solid food were then centrifuged at 7,000 × *g* for 30 min at 4°C to remove solid particles. Subsequently, the aqueous supernatant was removed and used for the assay.

ELISA. Microtiter plates (Immulon-4; Dynatech Laboratories, Chantilly, VA) were coated with affinity-purified capture antibody (100 μl/well) in a coating buffer (0.1 M Na_2CO_3, pH 9.6) at 2 μg/ml A, B, and E or 1 μg/ml F. A plastic plate seal was applied to the top of the plate and stored overnight at 4°C. The plates were washed and then blocked (200 μl/well) by adding 1% casein buffer, pH 7.4. After incubation for 30 min at 37°C, the blocking reagent was discarded, and the plate was tapped over absorbent paper to remove the remaining residual blocking casein. For the toxin standard curve, the botulinum neurotoxin complexes were prepared by diluting stock toxin solutions with the casein buffer. A spiked food sample, a negative control for each food type, or toxin standards (100 μl) were then added into the plate wells. After incubation for 2 h at 37°C, the plates were washed with 0.01 M PBS, pH 7.4, containing 0.005% of Tween 20. The DIG-labeled affinity-purified antibody was diluted 1:200 for type A and 1:400 for types B, E, and F in casein buffer, and 100 μl/well was dispensed to the plate and incubated at 35°C for 60 min. The horseradish peroxidase-conjugated antidigoxigenin antibody (antidigoxigenin-peroxidase [polyclonal], fab fragment), was diluted 1:5,000 in casein buffer; 100 μl/well was then added and incubated at 37°C for 60

min. After the washing step a substrate solution containing 3,3′5,5′-tetramethylbenzidine (a liquid substrate) was added into the wells (100 μl/well), and the plates were incubated at 37°C for 15 min in a dark chamber. The reaction was subsequently quenched with 1 N sulfuric acid (100 μl/well), and the color was monitored by measuring the absorbance at 450 nm on a Bio-Tek 311 Microtiter Plate Reader (Winooski, VT). A standard curve was constructed by plotting the absorbance values (mean of triplicate wells) against standard toxin concentrations, and unknown concentrations were determined from the linear regression equation.

Mouse bioassay. For food sample preparation, 10 g of spice samples were spiked with 200 ng of toxin for each BoNT complex A, B, E, or F in separate vials and incubated for 30 min at room temperature (25°C). The type E toxin was trypsinized before food samples were spiked. After incubation, the samples were mixed with 10 ml of gelatin phosphate buffer (2% gelatin in a 0.01 M sodium phosphate buffer, pH 6.2) and homogenized with a benchtop stomacher (Seward, Cincinnati, OH) to make a homogeneous suspension. The spice-buffer mixtures were then centrifuged at 7,000 × *g* for 30 min at 4°C to remove solid particles. Subsequently, the supernatant was diluted 1:10 with gelatin phosphate buffer in a glass test tube and was filter sterilized with a 0.45-μm-pore-size filter. Mice were injected intraperitoneally, in duplicate (0.5 ml/mouse), with the filtered food sample with or without toxin. Mice were observed for signs of botulism and death over a 48-h period. Toxicity was expressed as the number of hours until death.

RESULTS

Sensitivity and cross-reactivity. The sensitivity of the DIG-ELISA in casein buffer was 60.3 pg/ml (1.9 LD_{50}) for BoNT type A (BoNT/A), 176 pg/ml (1.58 LD_{50}) for BoNT/B, 163 pg/ml (4.5 LD_{50}) for BoNT/E, and 117 pg/ml for BoNT/F (less than 1 LD_{50}) in casein buffer (Fig. 1A and B). At the limit of sensitivity, the absorbance reading for positive samples was typically found to be twice that of the negative control. Accurate quantitation was possible at more than 2 ng/ml of neurotoxin in most food samples, since the absorbance level of a positive sample was typically three to four times higher than that of the negative control (casein buffer). The absorbance value variation between duplicate wells was typically 4 to 8%. Cross-reactivity at 10 ng of neurotoxin/ml was evaluated with each serotype-specific antibody. Each antibody type was found to detect its corresponding toxin type with less than 0.82% cross-reactivity between other toxin types (data not shown).

Detection of BoNTs in food samples. BoNT complex types A, B, E, and F were detected using the ELISA in a variety of liquid, semisolid, and solid food samples at 2 ng of toxin/ml. The results obtained are shown in terms of mean optical density at 450 nm (Fig. 2A and B and 3A and B). The assay detected the presence of A, E, and F in all food types except for a few spices and potato salad. Type B toxin was not detectable in spices and certain solid-meat-based samples such as smoked turkey, turkey breast, roast beef, cured ham, pepperoni, hard salami, cooked ham, and domestic Swiss cheese at 2 ng of toxin/g of food. The absorbance of the positive control for the toxin type B was found to be 44, 50, and 53% less compared to the positive control for types A, E and F, respectively. In additional experiments, we compared the ELISA with the mouse lethality test. The mouse assay was carried out with cumin, oregano, allspice, and cinnamon spiked with or without BoNT/A neurotoxin in a gel phosphate buffer. Mice injected with the spiked toxin (2 ng/ml) died within 48 h, while the mice injected with extract without the toxin survived.

Detection of BoNT/A in cultured filtrate. Toxins from cultures of types A, B, E, and F isolated from different environmental and clinical sources were used for ELISA testing. A panel of proteolytic A, B, and F strains and nonproteolytic B, E, and F strains was tested after a period of 24 h of growth. The culture filtrate from each isolate was diluted from 1×10^1 to 1×10^5 in casein buffer, pH 7.4, before testing by ELISA. Culture fluid dilutions above 1×10^5 were negative by ELISA. Seventy-two of the type A strains were ELISA positive at 1×10^5. Strains SKOR1-A(8933B1), CS3-A, JP2-A, JP8-A, and 4896-2 that were negative at 1×10^5 dilution were also ELISA negative at the 1×10^4 dilution. All of the culture filtrates for the 77 type A strains were positive at the 1×10^3 dilution, and all 12 strains of type B, E, and F were positive at 1×10^4 dilution. In order to test for false-positive results, we examined denatured botulinum neurotoxin complex types A, B, E, and F (heated at 100°C for 10 min), tap water, bovine serum albumin, purified Hn-33 (from botulinum neurotoxin complex), and culture filtrates of 26 other nonbotulinum strains including strains of *Clostridium acetobutylicum*, *Clostridium chauvoei*, *Clostridium sporogenes*, *Clostridium histolyticum*, *Clostridium tetani*, *Clostridium bifermentans*, *Clostridium sordellii*, *Clostridium perfringenes*, *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus megaterium* as interfering agents. No false positives were observed in culture filtrates of 26 strains or the other interfering agents.

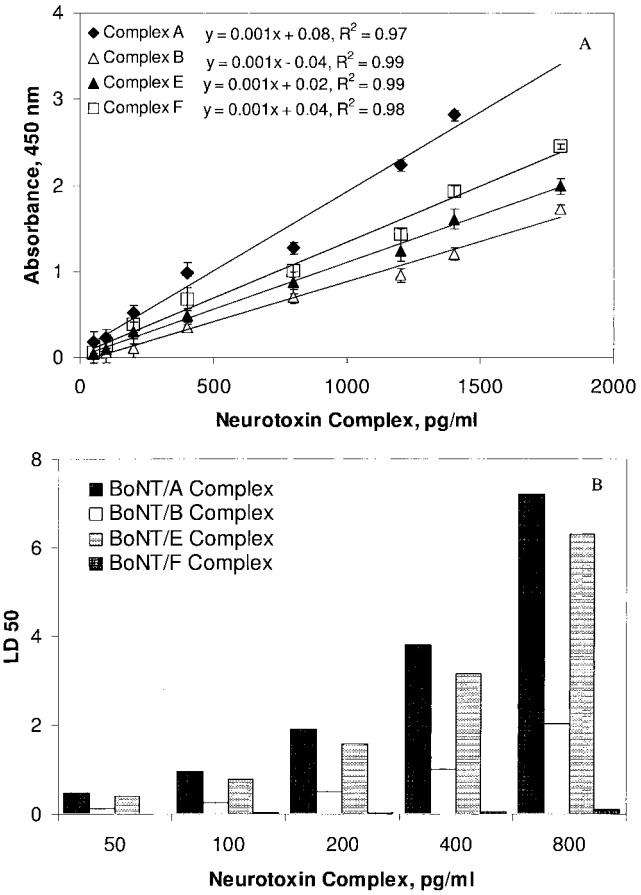

FIG. 1. Standard curve for the four botulinum neurotoxin complex serotypes in casein buffer. (A) Standard curve for BoNT/A, BoNT/B, BoNT/E, and BoNT/F. Points represent the mean absorbance values of duplicate wells. Where not visible, the error bars are within the symbol. (B) Relationship between mouse LD_{50} and concentration.

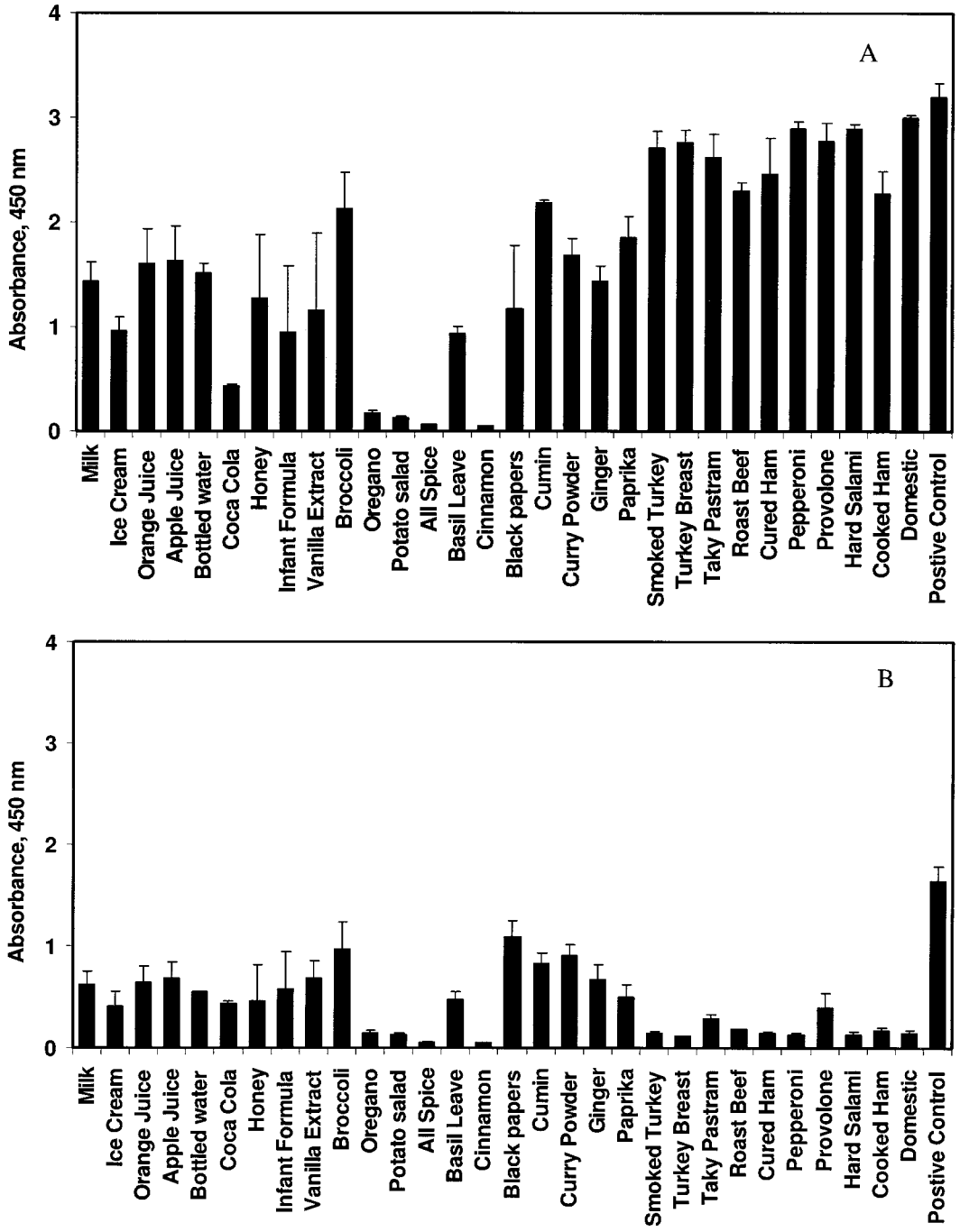

FIG. 2. The ELISA determinations of 2 ng/ml BoNT/A (A) and BoNT/B (B) complexes in food samples. The samples were spiked with neurotoxin complex and incubated at 30 min at room temperature. The absorbance of the negative food sample controls was subtracted from the positive food sample absorbance. The toxin extraction procedures are given in Materials and Methods.

DISCUSSION

The most sensitive and currently accepted method available for the measurement of biologically active toxin is the mouse bioassay. The mouse bioassay is still considered the standard method for toxin detection and serotyping. To avoid animal use, a search for alternative in vitro assays of similar sensitivity is necessary. Immunoassays for botulinum neurotoxin detection are capable of detecting as little as 10 to 100 minimum lethal doses/ml for type A toxin (8–11, 31, 48, 49). These in vitro methods were designed to virtually replace the mouse bioassay but have not been adequately validated for screening large numbers of samples. Ferreira et al. (16) reported the use of an amplified ELISA for detection of preformed type A toxin and culture toxins from hash brown potatoes associated with food-borne botulism.

The DIG-ELISA utilizes BoNT-specific antibodies labeled

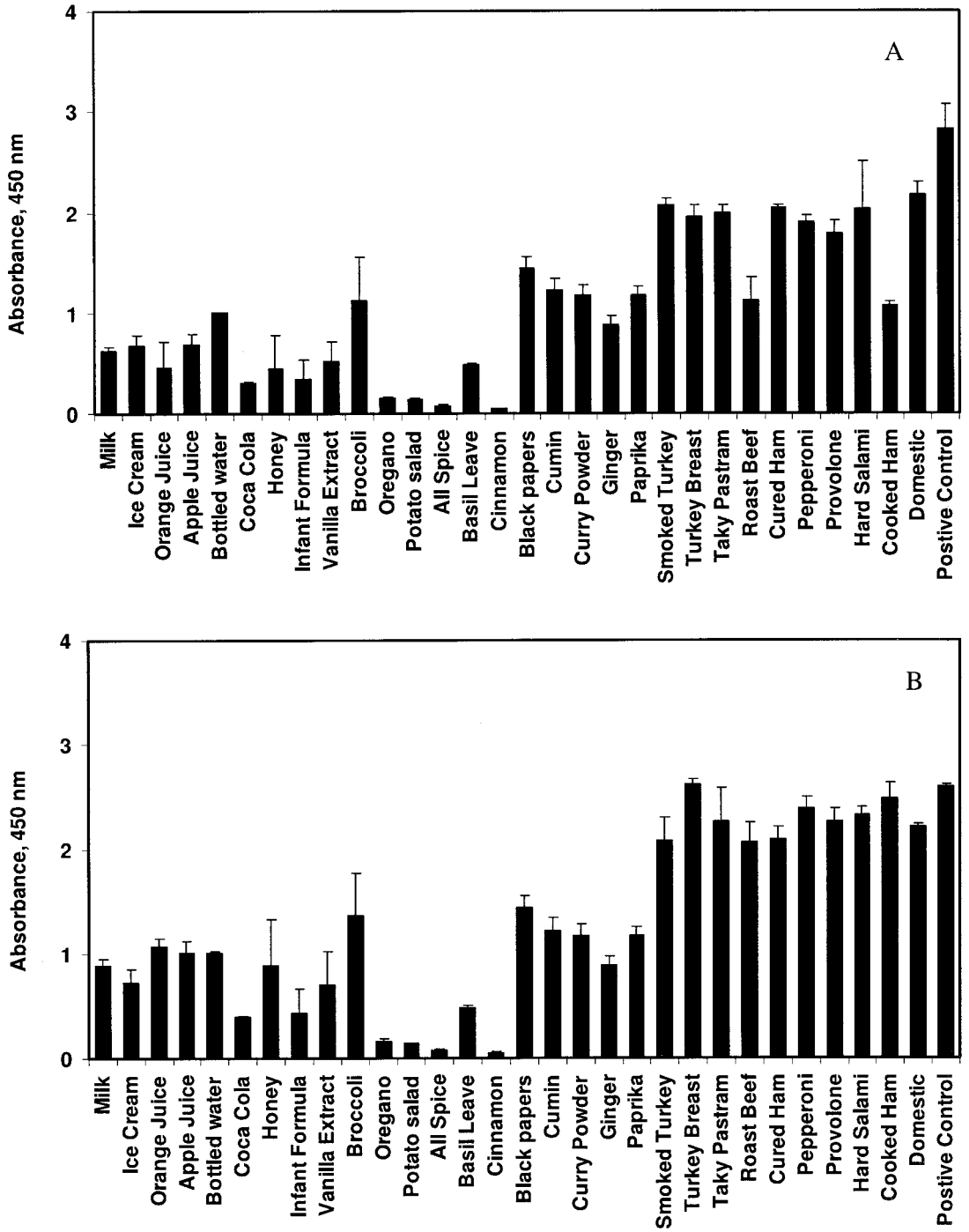

FIG. 3. The ELISA determinations of 2 ng/ml BoNT/E (A) and BoNT/F (B) complexes in food samples. The samples were spiked with neurotoxin complex and incubated for 30 min at room temperature. The absorbance of the negative food sample controls was subtracted from the positive food sample absorbance. The toxin extraction procedures are given in Materials and Methods.

with DIG. There are three features of the DIG molecule that make it exceptionally valuable for immunological detection systems. First, DIG can be coupled as a hapten to a suitable carrier molecule. High-affinity antibodies can be easily generated against the hapten or hapten carrier complex. Since DIG occurs exclusively in digitalis plants, there are no endogenous background problems with these antibodies such as occurs in the case of other haptens, such as biotin. Biotinylated antibodies are not used as a primary or capture source since the natural biotin found in certain foods such as milk may give background signals. Second, DIG can be coupled to antibodies, and the resulting antibody complex can be used in standard signal amplification procedures, including in situ hybridization, and can be detected with antidigoxigenin conjugates. Third,

the DIG system does not require learning a new technology by most food analysts who are familiar with ELISA methodologies.

The antibodies for BoNTs A, B, E, and F used in this assay were developed using the pure dichain BoNTs A, B, E, and F as antigen (a 150-kDa protein). Naturally occurring BoNTs, however, are complexed with nontoxic NAPs, which protect the dichain toxin from acidic and/or proteolytic digestion in the gastrointestinal tract (24, 31, 34, 37, 42, 43). Epitope mapping of type A complex BoNT suggests that the NAPs cover a large portion of the binding domain of the BoNT (5–7, 37). Therefore, these proteins could possibly block the antigenic site and prevent the recognition of the neurotoxin by the antibodies (31). We evaluated the assay with the neurotoxin complex to determine (i) if the assay could detect dichain botulinum neurotoxins in conjunction with their NAPs, especially in complex matrices such as foods, and (ii) if the pure dichain botulinum neurotoxin is relatively difficult to purify compared to the complex form. The former reason is particularly important since the complex neurotoxin is the form found in foods during *C. botulinum* growth.

This study was also used to assess the detection efficiency of the DIG-ELISA and its capacity for detecting low concentrations of BoNT complex (A, B, E, and F) in a variety of food matrices and in culture filtrates of *C. botulinum* strains. In our earlier report, we found that the assay sensitivity for purified dichain toxin and toxin complexed with NAPs was comparable (for mouse, <25 LD_{50}/ml) for types A, B, E, and F (1, 36). Our results in this study are consistent with the earlier report and suggest that the assay sensitivity is sufficient to easily detect botulinal toxin far below the human lethal oral dose, which is about 70 μg of toxin for 70 kg of body weight (2). It is important to understand the relationship between the concentration of toxins (typically given in pg/ml) which may be in dichain or complex form and the LD_{50} (Fig. 1 B). The presence of NAPs affects the toxin molecular mass calculations of complex compared to dichain toxin. The biological activity which is expressed in LD_{50} or sometimes minimal mouse lethal dose is more relevant to the measurement of toxicity and is dependent on a variety of factors including the purification method for toxins and perhaps how the toxin is produced in culture and by what strain of a given type. Although BoNTs exhibit about 40% amino acid and 50 to 60% sequence homology among various serotypes, the immunological cross-reaction among serotypes is minimal (22, 25, 40). Less than 1% cross-reactivity among antibodies has been reported in previous ELISAs (31). We also did not find any significant cross-reaction among antibodies even at a five-times-higher concentration of the neurotoxin (less than 0.8% cross-reactivity), indicating that polyclonal antibodies are specific and do not interfere with the applicability of the assay. The absorbance values from the cross-reaction experiments were 0.8% compared to the absorbance of a 1 μg/ml concentration of the neurotoxin complex control.

Sensitivity is always a major part of any detection system. Under ideal conditions, the desired sensitivity can be achieved if the sample does not contain substances that interfere with the detection system. Environmental and serum samples are ideal samples since they require little or no processing and can be used directly for detection. On the other hand, complex food matrices require processing and extraction of analyte before they can be used in assays. Moreover, contaminated food samples may cause cross-linking of immune complexes using protein A, protein G, or lectins (12, 30). Therefore, it is imperative that the assay be sufficiently robust to detect the lowest level of toxin in a variety of foods without any significant cross-reactivity or interference. We tested about 30 food samples consisting of liquid, semisolid, and solid foods. Types A, E, and F were detected in almost all food types except a few spices and potato salad. There were significant variations in the absorbance value at a given toxin concentration for each food type. The varied absorbance value for each food type could be due to several reasons. One of them would be matrix interference. Since each food type has a unique biochemical composition, it would be difficult to predict which component of the food may be interfering with the toxin complex or with the antigen-antibody interaction in the ELISA process. To test whether the toxin is destroyed or the immunoreaction is inhibited by spices, we used the mouse bioassay to determine the biological activity of the toxin in a few of these matrices (spices). The mouse bioassay results indicated that the spices may have exerted an inhibitory effect on the ELISA process but did not destroy the biologically active toxin. However, we were able to detect the toxin in spices and potato salad at a concentration of 5 ng/ml. It is probable that spices may inhibit the binding of toxin with antibody at a lower concentration of the toxin (2 ng/ml), although other explanations are possible. Additional research is under way to enhance the test for the detection of botulinum toxins in spices.

BoNT type B complex was not detected at 2 ng/ml of toxin complex in certain meat-based samples (Fig. 2B). However, the detection was possible at a concentration of 5 ng/ml. Since the absorbance of BoNT type B positive control was lower than BoNT complex types A, E, and F, it is possible that the BoNT complex type B antibody may not be reacting as well as other serotype antibodies to the toxin. Other antibody sources are being investigated that may overcome this problem. It is also possible that botulinum neurotoxin type B complex nonspecifically attaches to certain components of the meat products, which is difficult to discern since the components in the meat products are unknown.

The assay for this study was designed to test only undiluted or very low dilutions of culture filtrates. However, testing of higher dilutions revealed relatively high toxin levels in the cultures with positive results at the 1×10^4 dilution. Although the toxicity varies among strains of the same serotype, the type A toxin Hall strain typically produces 1 to 4 million LD_{50}/ml in culture (35). The assay detected toxin in diluted 24-h cultures at 1×10^4 dilution for a large panel of type A and at 1×10^3 for types B, E, E, and F strains. The possible cause of low toxicity is most likely the shortened culture time of 24 h compared to the usual 5 days of culture for toxin production. One of our objectives was to shorten the culture time to determine the rapid detection of toxin. This is particularly important in outbreak scenarios. Food samples may contain few spores or vegetative cells that require extended incubation, and culture methods to produce adequate toxin to be detected by the assays. The culture filtrate is a crude toxin containing neurotoxin and cellular proteins. Only five strains of type A cultures

were found negative at the maximum dilutions but detected at lower dilutions. To confirm suspected samples with false-negative reactions, the cultures should be incubated for a longer period of time.

The degree of toxicity varies among strains (12, 17), and because strains used in this study were isolated from different sources, epitope differences in a toxin could lead to weak or enhanced ELISA results. Certain nonbotulinum species such as *Clostridium butyricum* and *Clostridium baratii* also have been reported to produce BoNT (21, 27, 28, 41). Botulinum and tetanus neurotoxins also have several common features, including over 30% sequence homology with similar secondary structure content (3, 13, 15, 41, 45, 46). We tested cross-reactivity with culture filtrates of *C. butyricum* and *Clostridium tetani* along with other nonbotulinum strains and found no cross-reaction. Ferreira et al. (17) previously reported *Clostridium novyi* cross-reactivity using the amplified ELISA for types A, B, E, and F, albeit below the absorbance criteria judged to be positive. Although the assay format used in this study was different than the amplified ELISA, the antibodies were from the same sources. Our results show that *C. novyi* did not react with the assay. We found a 0% false-positive rate with the nonbotulinum toxin-producing organism tested.

In summary, we present a simple and sensitive ELISA that can be used as a preliminary screening test for the range of foods tested in this study and for types A, B, E, and F toxin-producing strains from culture medium. The number of samples that can be assayed using the mouse bioassay is severely restricted, and it may take up to 2 days for positive samples to be recognized. Although more extensive validation of the assay is required for a wider range of foods and for environmental and serum samples before it can be used as a screening system, we believe that the ELISA fulfills the current need for a rapid and robust detection system for BoNT types A, B, E, and F in toxin-contaminated foods.

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