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Methods for Detection of *Clostridium botulinum* Toxin in Foods†

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ABSTRACT

Botulism is a deadly disease caused by ingestion of the preformed neurotoxin produced from the anaerobic spore-forming bacteria *Clostridium botulinum.* Botulinum neurotoxins are the most poisonous toxins known and have been a concern in the food industry for a long time. Therefore, rapid identification of botulinum neurotoxin using molecular and biochemical techniques is an essential component in the establishment of coordinated laboratory response systems and is the focus of current research and development. Because of the extreme toxicity of botulinum neurotoxin, some confirmatory testing with the mouse bioassay is still necessary, but rapid methods capable of screening large numbers of samples are also needed. This review is focused on the development of several detection methods for botulinum neurotoxins in foods.

Botulinum toxins are the most toxic compounds known. The lethal dose for mice is 0.3 ng/kg, and the lethal dose for humans is thought to be 0.2 to 2.0 μg/kg *(45).* The seven different serotypes (A through G) of botulinum neurotoxin (BoNT), each produced by different strains of *Clostridium botulinum,* cause flaccid muscle paralysis by blocking the release of the neurotransmitter acetylcholine at neuromuscular junctions. No effective chemical antidote currently is available for botulism, primarily because of the lack of knowledge about the molecular structure and activity of BoNTs *(76).* Antitoxin can be given in certain cases of foodborne botulism but not in cases of infant botulism. Early administration of antitoxin limits the extent of paralysis but does not reverse it. Detection of *C. botulinum* neurotoxins has always been a major challenge for researchers because of the complex nature of their toxicity. To our knowledge, this is the only known example of a bacterial toxin in which a group of nontoxic proteins protects the neurotoxins from environmental extremes.

Comprehensive biochemical analysis of botulinum toxin is needed to understand the complex nature of its activity and to develop accurate detection techniques. Analytical techniques such as high-performance liquid chromatography *(3)* and mass spectrophotometry *(85, 86)* have been used for accurate and sensitive detection of toxin in high-output screening situations. Gas chromatography also has been used for the detection of toxin in food samples *(44).* Although these methods are reliable and sufficiently sensitive, they require special equipment, extended laboratory time, and trained personnel to carry out the complex analysis. Common obstacles in all currently available biosensor technologies, such as those using surface plasmon resonance *(35, 46),* fluorescence *(1, 47, 87),* chemiluminescence *(60, 92, 93),* and refractometry *(16, 38),* include lack of sensitivity, specificity, or repeatability when compared with the standard enzyme-linked immunosorbent assay (ELISA) and/or the more rapid assays such as hand-held assays (HHAs). The mouse bioassay remains the only validated method for food analysis of BoNTs, but promising new detection methods are being developed and are the subject of the this review.

ORGANISM

The genus *Clostridium* is defined as consisting of strictly anaerobic endospore-forming rods. Clostridia are widely distributed in soil, sewage, marine sediments, milk, fresh meat, and the intestines of humans and animals. Bacteria in this genus form spores and therefore are very resistant to heat treatment. BoNTs are produced by various strains of the anaerobic spore-forming bacilli: *C. botulinum, Clostridium butyricum,* and *Clostridium barati (4, 30, 74).* The seven serotypes (A through G) *(62)* are taxonomically divided into four groups *(13, 28, 34, 80).* Group I organisms are slightly curved rods with peritrichous flagella. They grow optimally at 37°C, are proteolytic, and produce A, B, or F toxins. Group II organisms are straight rods with peritrichous flagella. They grow optimally at 30°C, are not proteolytic, and produce B, E, or F toxins. The spores of *C. botulinum* type E have characteristic appendages that are randomly distributed over the entire surface of the spores. Group III organisms are straight rods and have peritrichous flagella. They grow optimally at 37 to 40°C, range from being slightly to nonproteolytic, and produce C1, C2, or D toxins. Group IV organisms are also straight rods with peritrichous flagella. They grow at 25 to 45°C, are proteolytic, and produce G toxin. All four groups form subterminal ellipsoidal spores. Humans are sensitive to type A, B, E, and F toxins. Because the preformed BoNT rather than the organism itself is the pathogenic agent, the first step in the toxicoinfection process is the ingestion and absorption of the BoNT at the intestinal wall. After absorption in stomach

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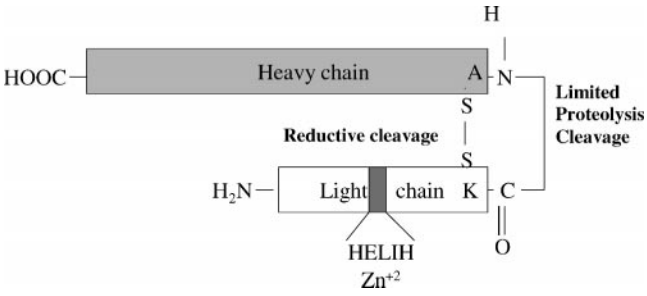

FIGURE 1. *Structure of botulinum neurotoxin type A showing common features of clostridial toxins. Adapted from (76).*

and intestine, the toxin is transferred to the neuromuscular junction, where it blocks acetylcoline.

TOXIN STRUCTURE AND FUNCTION

BoNTs are complexes of neurotoxin with one or more neurotoxin-associated proteins (NAPs), resulting in molecular sizes of 12S, 16S, and 19S *(58, 61, 82).* The 16S complex is referred to as the large (L) toxin and the 19S complex is referred to as the extra large (LL) toxin. The pure BoNT itself has been referred to as the small or 7S toxin *(58).* BoNT/A complex can exist in three forms: M, L, or LL *(61).* BoNT/B, BoNT/C, BoNT/D, and BoNT/E complexes exist in two forms: M and L *(42, 58, 94).* The BoNT/F complex is known to exist only in the M form, and the BoNT/G complex exists only in the L form *(23).* The M complex consists of the BoNT and the neurotoxin-binding protein *(77),* which is also commonly referred to as nontoxic nonhemagglutinin *(57).* The L complex consists of BoNT, the neurotoxin-binding protein, and five other NAPs. Genes for these proteins are topographically located in close proximity to each other on the bacterial DNA strand, and their expression is regulated in a coordinated manner *(15, 43).*

The number of NAPs differs in all seven serotypes. For example, there are seven NAPs in serotypes A and B and only one in serotype E *(58, 77).* However, Singh et al. *(78)* reported that type E serotype of the Alaska strain secretes five associated proteins. NAPs increase the toxicity of the neurotoxin and play a critical role in the toxicoinfection process *(6, 69).* For example, without NAPs the oral toxicity of the purified neurotoxin is decreased by 43,000-fold *(77).* NAPs protect neurotoxin against proteases, acidity, and heat *(40, 59, 67, 81),* and they also may be involved in translocating BoNT across the mucosal layer *(58).* However, a clear understanding of the translocation mechanism is lacking.

Each neurotoxin (7S) consists of a heavy chain (M_r approximately 100,000) and a light chain (M_r approximately 50,000) covalently linked by a single disulfide bond (Fig. 1). The heavy chain includes domains for binding to peripheral motor neurons and for toxin internalization, and the light chain is a zinc metalloprotease, specific for proteins involved in acetylcholine release. BoNT/A, BoNT/C, and BoNT/E cleave synaptosome-associated protein of M_r 25,000 (SNAP-25), and synaptobrevin or vesicle-associated membrane protein (VAMP) is the target of BoNT/B, BoNT/D, BoNT/F, and BoNT/G. In addition to SNAP-25, BoNT/C also cleaves syntaxin (Fig. 2 and Table 1). Each neurotoxin hydrolyzes only a single bond in its substrate, which is sufficient to prevent exocytosis of neurotransmitter *(36, 62).* The mode of action of BoNT can be divided into four steps: ingestion and intestinal absorption, binding to neuronal membranes, internalization, and blockage of neurotransmitter release *(18).*

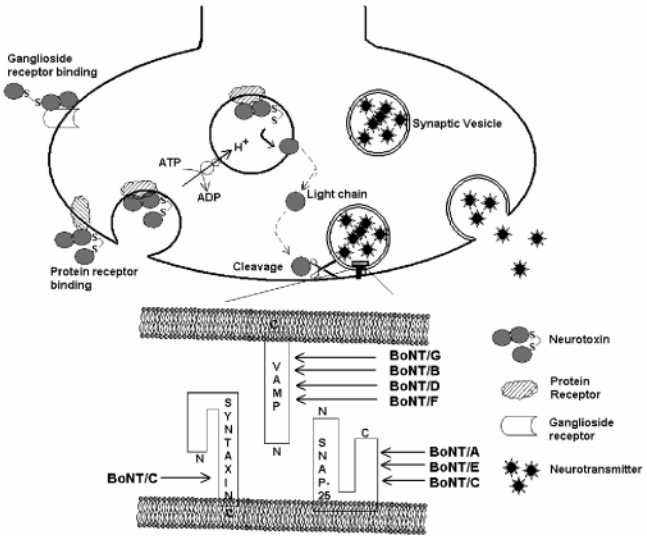

FIGURE 2. *Model of the neurotransmitter release and action of botulinum toxin. Botulinum toxin binds to the presynaptic membrane receptor mediating endocytosis. The light chain is translocated across the membrane and acts as a specific endopeptidase (inset) and cleaves the substrate before the SNARE complex is formed. Adapted from (76).*

DETECTION

There are several methods available for the toxin detection (Table 2). Of all the methods for identification of *C. botulinum* and detection of its toxin in foods, the intraperitoneal injection of mice remains the most reliable, sensitive, and definitive. The presence of the toxin is detected by injection of the food extract into mice, which are then observed for characteristic symptoms of botulism and ultimately death over a 48-h period. The mouse bioassay is

TABLE 1. *Exclusive substrate for botulinum neurotoxins in neuronal cells*

Serotype	Substrate[a]	Reference(s)
BoNT/A	SNAP-25	*68, 69*
BoNT/B	VAMP/synaptobrevin	*64*
BoNT/C	Syntaxin	*5*
BoNT/D	VAMP/synaptobrevin cellubrevin	*63*
BoNT/E	SNAP-25	*68*
BoNT/F	VAMP/synaptobrevin cellubrevin	*63*
BoNT/G	VAMP	*65*

[a] SNAP-25, synaptosome-associated protein of 25 kDa, a soluble protein that plays a key role in vesicle membrane fusion events with the plasma membrane; VAMP, vesicle-associated membrane protein or synaptobrevin. Cellubrevin is a member of the synaptobrevin/VAMP family.

TABLE 2. *Detection methods for* C. botulinum *neurotoxin and their detection limits*

Method	Format	Detection limit		Time required (including sample preparation)	Reference(s)
		ng/ml	MLD_{50} unit/ml		
Mouse bioassay	In vivo, functional	0.01	1	48 h	*88*
ELISA	Immunoassay	0.1–1	10–100	8 h	*21*
Hand-held assay	Immunoassay	10–20	1,000–2,000	15 min	
ORIGEN[a]	Chemluminescent	0.05–0.1	5–10	2 h	*24, 25*
Functional assay	Peptide substrate	0.005	~1	4–5 h	*88*

[a] From Igen Technology, now BioVeris Corporation.

the only standard validated method for the detection of botulinum toxin in foods *(8).*

Comparisons between the mouse bioassay and current in vitro tests for botulinum neurotoxins can be made to identify the relative strengths and weakness of the in vitro tests. Major limitations of the in vitro assays are the lack of quantitative correlation and poor sensitivity compared with the in vivo mouse bioassay *(70).*

Mouse bioassay. In the mouse bioassay, the biological activity of freshly prepared toxin from food samples is quantified by comparing its potency to that of a standard (sensitivity, 10 pg of the toxin). This method causes pain and suffering to animals and, therefore, should not be used without justification. The assay is severely limited because a single sample requires 6 to 10 min of individual preparation time, 6 to 16 mice, and up to 2 days for positive samples to be recognized. The concentration of the toxin can be estimated by serial dilutions of the toxin and injection into multiple additional mice. In the mouse assay, the presence of functionally active neurotoxin is recognized by specific symptoms or death in the mice. Typical botulism symptoms in mice sequentially consist of ruffled fur, labored but not rapid breathing, weakness of the limbs progressing to total paralysis, gasping for breath (opening of lower jaw), and finally death due to respiratory failure. Toxins type can also be determined by using mice protected with a monovalent type of specific antitoxin serum administrated 30 min before injection with the toxin. This method has been used successfully by the U.S. Food and Drug Administration (FDA), Centers for Disease Control and Prevention, and state public health laboratories to identify the causative food and toxin type associated with cases of botulism.

A variation of the mouse bioassay in which only one mouse is needed was developed to test the toxicity of BoNT *(70).* However, this assay is considered an in vitro assay. Intact hemidiaphragms and attached phrenic nerves are removed from the mouse, and the nerve-muscle preparations are mounted in a tissue bath. The phrenic nerve is then stimulated with single supramaximal pulses. The resulting muscle twitches are measured with an isometric force transducer, and the data are stored and analyzed by computer. The peak isometric tension following each stimulus is converted to the fraction of the control twitch tensions obtained before addition of the toxin. The efficacy of the assay was compared with the classical mouse survival assay. In some experiments, the sensitivity was not equal to that of mouse survival assay. However, in studies in which toxin was neutralized with equine antiserum, the in vitro muscle assay was three times more sensitive to the presence of antiserum than was the equivalent mouse survival assay. This assay can be used in the development of new drugs to treat botulism where the antagonistic sensitivity is a primary consideration in determining relative efficacy during structural activity studies.

ELISA. The ELISA is used to measure interaction between antigen and antibody and can be used for detection of a variety of protein antigens from pathogenic organisms. There are five basic steps in the ELISA procedure. First, the wells of a plastic microtiter plate are coated with a suitable concentration of antibody against each protein antigen of the pathogen. The antibody is strongly adsorbed by the plastic and remains attached to the well surface throughout the rest of the procedure. In the second step, any sites on the plastic surface still capable of binding protein are blocked by addition of an unrelated protein such as gelatin, bovine serum albumin, or nonfat dried milk. The application of blocking proteins reduces nonspecific binding of the primary or secondary antibodies to sites in the microtiter plate wells. The excess blocking protein is then removed by washing the plates with suitable buffer. In the third step, food sample extracts containing toxin antigen are added to the wells so that any toxin antigen present in the samples can bind to the antibody. The remaining antigen and other proteins present in the sample are then removed by washing the plate with buffer. In the fourth step, the bound antigen is detected by the addition of an enzyme-conjugated antibody, which binds to the antigen but does not react with the blocking protein. There are separate antibodies for each toxin type (A, B, E, and F), and each toxin type should analyzed separately. In the fifth step, the substrate for the enzyme bound to the antibody is added to the wells. The amount of enzyme-conjugated antibody bound in each well is measured by the ability of the enzyme to hydrolyze a colorless substrate to yield a colored product, and the color intensity is quantitatively measured in a specially adapted spectrophotometer (microplate reader). Several modifications, such as direct or indirect antigen-antibody interaction signal amplification by enzyme or substrate manipulation, can enhance the sensitivity and repro-

ducibility of the methods. However, the basic principle of protein-protein (antibody-antigen) interaction remains the same in almost all ELISAs.

The high affinity and specificity of avidin-biotin and antibody-hapten interactions have been exploited for diverse applications in immunology, histochemistry, affinity chromatography, and many other areas *(11).* Biotinylation provides the ''tag'' that transforms molecules that are difficult to detect into probes that can be recognized by a labeled detection reagent or an affinity-capture matrix. Once tagged with biotin, a molecule of interest, such as an antibody, can be used to probe toxins. This tagged molecule can then be detected with the appropriate avidin or antihapten antibody conjugate that has been labeled with an enzyme. An amplified ELISA that uses the biotin-streptavidin system was developed and compared with standard Association of Official Analytical Chemists methods for detection of *C. botulinum* toxins. This amplified ELISA was used to screen suspect cultures for botulinal toxins in food *(19, 20, 22).* The total time required to run a set of samples by ELISA, including sample preparation, is approximately 5 to 6 h. Validations of the ELISA detection assay were reported by several laboratories *(10, 52, 72),* but these assays lacked the sensitivity of the mouse bioassay.

An enzyme-linked coagulation assay (ELCA) was reported to be as sensitive as the mouse bioassay *(14).* This assay is highly sensitive, but it relies on a sophisticated amplification system utilizing a snake venom coagulation factor and is limited by its complexity and the expense of the reagents. In this assay, a complex consisting of toxin bound to chicken antibody (or biotinylated antibody) and an antibody labeled with Russell's viper venom factor X activator is formed in a solution phase. The solution phase complex is then captured onto a plate coated with anti-chicken immunoglobulin G or avidin, and the resulting color is measured.

Although ELCA is highly sensitive, it has limitations for food testing. The combination of antibodies (chicken antibodies and biotinylated 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). Nevertheless, Roman et al. *(55)* successfully used ELCA to detect botulinum toxin type E in fish.

A sequence comparison revealed 40% amino acid and 50 to 60% nucleotide sequence homology among various serotypes of BoNT *(79).* The antibody against the heavy chain C-fragment may be less subject to binding interference or nonspecific binding than those antibodies directed against whole toxin. Poli et al. *(52)* developed a modified ELISA for serotypes E and F using affinity-purified horse polyclonal antibodies directed against the 50-kDa C-fragment. The sensitivities of the assay were 0.5 ng/ml for BoNT/E and 2 ng/ml for BoNT/F in the assay buffer. A similar approach was employed by Szilagyi et al. *(83)* for serotypes A and B. Detection was possible at 20 pg per well, and accurate quantitation was obtained at 50 pg per well. These assays are good examples of simple colorimetric ELISAs that can be used to detect minute toxin concentration.

All the serotypes of BoNT are immunogenic and can evoke neurotoxin-neutralizing antibodies *(7, 41).* Although polyclonal antibodies in general do not cross-react among different serotypes of BoNT (except between C1 and D and between E and F) *(41),* certain monoclonal antibodies and peptide-specific antibodies react with several serotypes of the neurotoxin *(32, 83, 84).* Both polyclonal and monoclonal antibodies were used with ELISAs for the detection and typing of the neurotoxins *(27, 48, 53, 54, 71, 78, 83).* Where comparative studies of monoclonal and polyclonal antibodies were conducted, polyclonal antibodies were more effective in detecting the neurotoxin *(48, 50).* Polyclonal antibodies have also been used in modified ELISAs such as the amplified ELISA *(21, 53),* the ELISA with ELCA *(14),* and the ELISA with passive hemagglutination assay *(33, 37).*

Recently, the FDA developed a sensitive and specific ELISA to detect *C. botulinum* neurotoxins A, B, E, and F. The assay uses polyclonal antibodies specific for each toxin type as both capture and digoxigenin-labeled detector molecules. The sensitivity of the assay was less than 60 pg/ml for all toxin types. Assay sensitivity to purified toxin and toxin complexed to NAPs was comparable ($<$25 mouse LD_{50}/ml) for types A, B, E, and F *(2).*

Lateral flow device. Immunochromatographic assays, also called lateral flow tests or simply strip HHAs, are rapid detection tests. The best example of such a test is that for early pregnancy detection in which the presence of human chorionic gonadotropin (hCG) is detected by anti-hCG antibodies. These tests are a logical extension of the technology used in latex agglutination tests, the first of which was developed in 1956 by Singer and Plotz *(75).* The benefits of immunochromatographic tests include (i) user-friendly format, (ii) very short turnaround time for results (typically 15 min), (iii) suitability for field use and where sophisticated equipment is not available, (iv) stability of test kit over time and over a wide range of climatic conditions, and (v) relatively inexpensive.

In principle, any ligand that can be bound to a visually detectable solid support, such as dyed microspheres, can be qualitatively and in many cases semiquantitatively tested. In these assays, a visible line is formed on a test strip when a liquid containing a particular biological agent of interest is applied to a test well. The liquid sample migrates via capillary action along the nitrocellulose membrane. A positive sample reacts with the agent-specific labeled antibody conjugate that is bound to the membrane and forms a visible line. In the absence of the specific agents, no line will develop where the conjugate is bound, indicating a negative result.

Limited literature is available on validation of HHAs compared with other established immunology-based and ELISA detection methods. This lack of interest may be because HHAs have sensitivities of 10 to 20 ng/ml of toxin and thus are less sensitive than the ELISA or mouse bioassays. However, these HHAs have been used successfully

to detect neurotoxins in a wide variety of foods, suggesting that they may still be useful as a preliminary screening system for triaging food samples with elevated BoNT concentrations in the event of a *C. botulinum* contamination event *(66).* Although these devices appear to be ideal, they have limitations. Their limits of detection are many fold higher than the toxic dose. They can also give false-positive results because of contaminants in the food. Therefore, the appropriate use of HHAs requires a thorough understanding of their limitations in sensitivity and capability. Most important, the user must have a preplanned series of confirmatory tests and follow-up assays when the device gives a positive result *(17).*

Immunofluorescence assay and biosensors. In recent years, various sensor technologies have been developed and tested for detection of foodborne pathogens *(12),* including botulinum toxins *(50, 79).* Biosensors, defined as analytical devices combining the molecular recognition capabilities of biomolecules with electronics for signal measurement, continue to capture the imagination of an ever-increasing number of scientists and engineers.

Ogert et al. *(50)* developed a fiber optic–based biosensor utilizing the evanescent wave of a fiber optic wave guide in combination with a sandwich immunoassay at the surface of the tapered optical fiber. In this format, the assay relies on the formation of a florescent complex of the botulinum toxin, an immobilized capture antibody, and a fluorescent antibody. By utilizing the evanescent wave of the fiber, the binding events along the surface of the tapered fiber can be transduced as an increase in fluorescence intensity. This biosensor technique is unique because only one step is required for the rapid detection of the botulinum toxin and continuous monitoring can be done. The sensor detected botulinum toxin A at 1 ng/ml. By careful selection of immobilized antibody, a set of sensors can be constructed that are specific and sensitive for each botulinum serotype.

Shriver-Lake et al. *(73)* further modified the fiber optics biosensor by employing fluorescence-based assays to improve signal-to-background ratios over those achievable with absorbance-based assays. Both distal and evanescent-wave fiber-optic fluorescence sensors were developed for the detection of large molecules *(89).* Evanescent-wave sensors have a particular advantage for fluorescence assays. The characteristic penetration depth of the evanescent wave provides a spatial separation of fluorescence signals associated with bound and free complexes. The long optical fibers facilitate easy handling of hazardous materials. Detection limits for botulinum toxins using this type of sensor were approximately 0.02 nM. A sensor that offers specificity and can detect all serotypes simultaneously has been developed by Rowe-Taitt and coworkers *(56).* In this type of sensor, a simple patterned array of antibodies is immobilized on the surface of a planar waveguide that captures antigens (BoNT) that are then bound by fluorescent tracer antibodies. Upon excitation of the fluorophore complex by a small diode laser, a charge couple device camera detects the assay pattern of the fluorescent antibody–antigen complexes on the waveguide surface. This array biosensor was used to detect botulinum toxin (toxins and toxoids) at 50 ng/ml in the samples.

ECL. Recent advances in electrochemiluminescence (ECL) detection were incorporated in the ORIGEN immunoassay system (Igen, Inc., Rockville, Md., now BioVeris Corporation). The technology offers the advantage of magnetic bead antigen capture and concentration from a variety of complex biological matrices. Unlike the typical ELISA in which either antibodies or antigens are directly bound to a polystyrene or polyvinyl microplate, antibodies are attached on the magnetic particles in a large volume of sample suspension and then captured and bound in a small area. ECL detection provides increased sensitivity because of high luminescent signal-to-noise ratios *(26, 39, 90–92).*

There are several advantages to an immunoassay using magnetic beads compared with the traditional ELISA: (i) potentially larger surface area *(9, 29, 49, 93)* on the beads provides for high density of antibody immobilization, (ii) kinetics of the antibody-antigen reaction in suspension is enhanced because the beads are not fixed *(51),* and (iii) high throughput. Moreover, a large number of food or environmental samples can be tested in a very short period of time when the sample processing and analysis procedures occur simultaneously *(24, 25, 91).* Although the immunomagnetic assay has been used for the detection of botulinum toxin in a variety of samples *(24, 25),* this assay has not been validated in a wide variety of food products, particularly solid and semisolid foods. Despite its unique technology, this system has received limited development for botulinum toxins. However, considering its use for several other biothreat agents, it is a promising method for detection of botulinum toxin in food.

Functional assay. The functional role of neurotoxin has opened new avenues for neurotoxin detection. Because BoNTs block neurotransmitter release by selective cleavage of synaptic proteins, an assay could be based on the detection of biologically active toxin. At present, the mouse bioassay is the only validated method for detection of biologically active toxin. In other in vitro methods, the presence of toxin molecules is measured by immunological methods, but the toxin may not necessarily be active, for example if the food had been heated. Attempts have been made to use an appropriate peptide substrate for quantifying the proteolytic activity of each neurotoxin. Schmidt et al. *(65)* developed high-throughput solid-phase protease activity assays for serotypes A, B, D, and F. Each assay consists of a cleavable oligopeptide based on natural substrate sequence that is labeled with fluorescein and covalently attached to a multiwell plate. When present, the toxin cleaves the oligopeptides and releases the fluorescein molecules. A toxin concentration of 2 ng/ml gave reproducible positive results. Hallis et al. *(31)* developed a solid-phase immobilized peptide-based assay system for the detection of BoNT/B based on the cleavage of a synthetic peptide substrate representing amino acid residues 60 to 94 of the intracellular target protein VAMP. In this assay system, immobilized VAMP peptide substrate (amino acids 60 to 94) is cleaved by BoNT/

B at the Gln-76–Phe-77 bond, leaving the C-terminal cleavage fragment bound on the solid phase. This fragment is then detected by the addition of an antibody-enzyme reagent that specifically recognizes the newly exposed N-terminus of the cleavage product. This assay is specific to BoNT/B, has no cross-reactivity with other clostridial neurotoxins, and has a sensitivity of 0.6 to 4.5 ng/ml. Although such assays are specific and include an estimate of the biological activity of the neurotoxin, they do not match the sensitivity of the mouse bioassay.

To improve sensitivity, Wictome et al. *(88)* developed an assay in which the mixture of cleaved and uncleaved peptides is first immobilized onto streptavidin-coated microtiter plates and the cleaved peptide is then detected by a cleavage product–specific antibody. Sensitivity exceeded that of the mouse bioassay (1 mouse LD_{50}), and the assay was sufficiently robust to detect BoNT/B in various food matrices such as paté, cheese, cod, and minced beef. The endopeptidase assay was approximately fourfold more sensitive than the mouse bioassay for BoNT/B, and the presence of food extracts (paté and sausage) had little effect on performance. Although this assay is specific for serotype B, it has potential for development because of its high sensitivity (comparable to that of the mouse bioassay) and excellent performance in foods where the food matrix poses an inherent obstacle to toxin detection.

SUMMARY

During the past 5 years, significant progress has been made in deciphering the mode of action of BoNTs, primarily because botulinum toxin is being used as a therapeutic agent. In the mouse bioassay, only a limited number of samples can be analyzed at one time. Therefore, there is a critical need for new rapid diagnostics that can be validated as substitutes for the mouse bioassay. This need is particularly urgent for detection of the toxin in situations where there are large numbers of samples. Although potential in vitro methods, including the digoxigenin ELISA, may be useful as preliminary screening tools, further evaluation of these assays for their performance with foods and clinical specimens is required before they can be considered effective for large-scale screening of the food supply. Development of biosensors, especially multichannel immunosensors, offers the promise of simultaneous detection of the four serotypes of *C. botulinum* and other threat agents. An interdisciplinary approach to research on the diagnostics of botulinum toxins in food is urgently needed.

REFERENCES

1. Anderson, G. P., K. A. Breslin, and F. S. Ligler. 1996. Assay development for a portable fiber optic biosensor. *Am. Soc. Artif. Intern. Organs J.* 42:942–946.
2. Andreadis, J. D., J. J. Kools, J. K. Dykes, J. L. Ferreira, and S. E. Maslanka. 2003. Development of an in vitro DIG-ELISA assay for detection of *Clostridium botulinum* neurotoxin serotypes A, B, E, and F. Poster presented at the first American Society for Microbiology Biodefense Research Meeting, Baltimore, 9 to 12 March 2003. Available at: http://www.asmbiodefense.org/2003tueabs.asp#172. Accessed 24 January 2005.
3. Anne, C., F. Cornille, C. Lenoir, and B. P. Roques. 2001. High-throughput fluorogenic assay for determination of botulinum type B neurotoxin protease activity. *Anal. Biochem.* 291:253–261.
4. Aureli, P., L. Fenicia, B. Pasolini, M. Gianfranceschi, L. M. McCroskey, and C. L. Hatheway. 1986. Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. *J. Infect. Dis.* 154:207–211.
5. Blasi, J., E. R. Chapman, S. Yamasaki, T. Binz, H. Niemann, and R. Jahn. 1993. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.* 12:4821–4828.
6. Cai, S., H. K. Sarkar, and B. R. Singh. 1999. Enhancement of the endopeptidase activity of botulinum neurotoxin by its associated proteins and dithiothreitol. *Biochemistry* 38:6903–6910.
7. Cardella, M. A. 1964. Botulinum toxoids, p. 113–130. *In* K. H. Lewis and K. Cassel, Jr. (ed.), Botulism: proceedings of a symposium. Public Health Service, U.S. Department of Health, Education, and Welfare, Cincinnati.
8. Center for Food Safety and Applied Nutrition. 2001. Bacteriological analytical manual (BAM). U.S. Food and Drug Administration, Washington, D.C.
9. Cudjoe, K. C., L. I. Thorsen, T. Sorensen, J. Reseland, Ø. Olsvik, and P. E. Granum. 1991. Detection of *Clostridium perfringens* type A enterotoxin in facial and food samples using immunomagnetic separation (IMS)-ELISA. *Int. J. Food Microbiol.* 12:313–316.
10. Dezfulian, M., and J. G. Bartlett. 1984. Detection of *Clostridium botulinum* type A toxin by enzyme-linked immunosorbent assay with antibodies produced in immunologically tolerant animals. *J. Clin. Microbiol.* 19:645–648.
11. Diamandis, E. P., and T. K. Christopoulos. 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. *Clin. Chem.* 37:625–636.
12. Dimitri, I., I. Abdel-Hamid, P. Atanasov, and E. Wilkins. 1999. Biosensors for detection of pathogenic bacteria. *Biosensors Bioelectron.* 1:599–624.
13. Doddes, K. L., and J. W. Austin. 1997. *Clostridium botulinum*, p. 288–304. *In* M. P. Doyle (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
14. Doellgast, G. J., M. X. Triscott, G. A. Beard, J. D. Bottoms, T. Cheng, B. H. Roh, M. G. Roman, P. A. Hall, and J. E. Brown. 1993. Sensitive enzyme-linked immunosorbent assay for the detection of *Clostridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. *J. Clin. Microbiol.* 31: 2402–2409.
15. East, A. K., M. Bhandari, J. M. Stacey, K. D. Campbell, and M. D. Collins. 1996. Organization and phylogenetic interrelationships of genes encoding components of the botulinum toxin complex in proteolytic *Clostridium botulinum* types A, B, and F: evidence of chimeric sequences in the gene encoding the nontoxic nonhemagglutinin component. *Int. J. Syst. Bacteriol.* 46:1105–1112.
16. Edwards, P. R., P. A. Lowe, and R. J. Leatherbarrow. 1997. Ligand loading at the surface of an optical biosensor and its effect upon the kinetics of protein-protein interactions. *J. Mol. Recognit.* 103:128–134.
17. Emanuel, P. A., C. Chue, L. Kerr, and D. Cullin. 2003. Validating the performance of biological detection equipment: the role of the federal government. *Biosecur. Bioterror. Biodefense Strat. Pract. Sci.* 1:131–137.
18. Eswaramoorthy, S., D. Kumaran, and S. Swaminathan. 2002. A novel mechanism for *Clostridium botulinum* neurotoxin inhibition. *Biochemistry* 41:9795–9802.
19. Ferreira, J. L., S. J. Eliasberg, P. Edmonds, and M. A. Harrison. 2004. Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinal toxin in food. *J. Food Prot.* 67:203–206.
20. Ferreira, J. L., S. J. Eliasberg, M. A. Harrison, and P. Edmonds. 2001 Detection of preformed type A botulinal toxin in hash brown potatoes by using the mouse bioassay and a modified ELISA test. *J. AOAC Int.* 84:1460–1464.
21. Ferreira, J. L., S. Maslanka, and J. Andreadis. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-

labeled IgGs and the ELISA. Department of Health and Human Services, Office of Regulatory Affairs Laboratory Information Bulletin no. 4292(18), p. 1–10. U.S. Food and Drug Administration, Washington, D.C.

22. Ferreira, J. L., S. Maslanka, E. Johnson, and M. Goodnough. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study. *J. AOAC Int.* 86:314–331.
23. Fujita, R., Y. Fujinaga, K. Inoue, H. Nakajima, H. Kumon, and K. Oguma. 1995. Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. *FEBS Lett.* 376:41–44.
24. Gamez, F., V. Rivera, J. White, and M. Poli. 2003. A sensitive and rapid electrochemiluminescent immunnoassay for the detection of *Clostridium botulinum* neurotoxins A, B, E and F in food matrices. Presented at the 40th Annual Interagency Botulism Research Coordinating Committee Meeting, Decatur, Ga.
25. Gamez, F., V. Rivera, J. White, and M. Poli. 2004. A sensitive and rapid electrochemiluminescent immunoassay for the detection of *Clostridium botulinum* neurotoxins A, B, E and F in food matrices. Presented at the 104th General Meeting of the American Society for Microbiology, New Orleans, 23 to 27 May.
26. Gatto-Menking, D. L., H. Yu, J. G. Bruno, M. T. Goode, M. Miller, and A. W. Zulich. 1995. Sensitive detection of biotoxoids and bacterial spores using an immunomagnetic electro-chemiluminescence sensor. *Biosens. Bioelectron.* 10:501–503.
27. Gibson, A. M., N. K. Modi, T. A. Roberts, C. C. Shone, P. Hambleton, and J. Melling. 1987. Evaluation of monoclonal antibody–based immunoassays for detecting type A *Clostridium botulinum* toxin produced in pure culture and inoculated model cured meat system. *J. Appl. Bacteriol.* 63:217–226.
28. Gimenez, D. F., and J. A. Gimenez. 1993. Serological subtypes of botulinual neurotoxin, p. 421–431. *In* B. R. DasGupta (ed.), Botulinum and tetanus neurotoxins. Plenum Press, New York.
29. Gunderson, S. G., I. Haagensen, T. O. Jonassen, K. J. Figenschau, N. de Jonge, and A. M. Deelder. 1992. Magnetic bead antigen capture enzyme-linked immunoassays in microtitre trays for rapid detection of schistosomal circulating anodic antigen. *J. Immunol. Methods* 148:1–8.
30. Hall, J. D., L. M. McCroskey, B. J. Pincomb, and C. L. Hatheway. 1985. Isolation of an organism resembling *Clostridium barati* which produces type F botulinal toxin from an infant with botulism. *J. Clin. Microbiol.* 21:654–655.
31. Hallis, B., B. A. James, and C. C. Shone. 1996. Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. *J. Clin. Microbiol.* 34:1934–1938.
32. Halpern, J. L., L. A. Smith, K. B. Seamon, K. A. Groover, and W. H. Habig. 1989. Sequence homology between tetanus and botulinum toxins detected by an antipeptide antibody. *Infect. Immun.* 57:18–22.
33. Hatheway, C. L. 1993. *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin, p. 3–20. *In* A. H. W. Hauschil and K. L. Dos (ed.), *Clostridium botulinum*: ecology and control in food. Marcel Dekker, Inc., New York.
34. Hatheway, C. L., and J. L. Ferreira. 1996. Detection and identification of *Clostridium botulinum* neurotoxins, p. 481–498. *In* B. R. Singh and A. Tu (ed.), Natural toxins II: structure, mechanism of action and detection. Plenum Press, New York.
35. Hsieh, H. V., B. Stewart, P. Hauer, P. Haland, and R. Cambell. 1998. Measurement of *Clostridium perfringens* beta-toxin production by surface plasmon resonance immunoassay. *Vaccine* 16:997–1003.
36. Humeau, Y., F. Doussau, N. J. Grant, and B. Poulain. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie* 82:427–446.
37. Johnson, H. M., K. Brenner, A. Angelotti, and H. E. Hall. 1966. Serological studies of types A, B, and E botulinal toxins by passive hemagglutination and bentionite flocculation. *J. Bacteriol.* 91:967–974.
38. Kenmore, C. K., S. R. Erskine, and D. J. Bornhop. 1997. Refractive-index detection by interferometer backscatter in packed-capillary high-performance liquid chromatography. *J. Chromatogr.* 762:219–225.
39. Kijek, T. M., C. A. Rossi, D. Moss, R. W. Parker, and E. A. Henchal. 2000. Rapid and sensitive immunomagnetic-electrochemiluminescent detection of staphylococcal enterotoxin B. *J. Immunol. Methods.* 236:9–17.
40. Kitamura, M., S. Sakaguchi, and G. Sakaguchi. 1969. Significance of 12S toxin of *Clostridium botulinum* type E. *J. Bacteriol.* 3:1173–1178
41. Kozaki, S., Y. Kamata, M. Takahashi, T. Shimizu, and G. Sakaguchi. 1989. Antibodies against botulinum neurotoxin, p. 301–318. *In* L. L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, San Diego, Calif.
42. Li, B., S. N. Parikh, R. B. Lomenth, and B. R. Singh. 1997. A novel type E *Clostridium botulinum* neurotoxin progenitor complex. *Protein Sci.* 6(Suppl. 2):139T.
43. Marvaud, J. C., M. Gilbert, K. Inoue, V. Fujinaga, K. Oguma, and M. R. Popoff. 1998. *botR* is a positive regulator of botulinum neurotoxin and associated nontoxic protein genes in *Clostridium botulinum* A. *Mol. Microbiol.* 29:1009–1018.
44. Mayhew, J. W., and S. L. Gorbach. 1975. Rapid gas chromatographic technique for presumptive detection of *Clostridium botulinum* in contaminated food. *Appl. Microbiol.* 29:297–299.
45. McGrath, S., J. S. Dooley, and R. W. Haylock. 2002. Quantification of *Clostridium botulinum* toxin gene expression by competitive reverse transcription-PCR. *Appl. Environ. Microbiol.* 66:1423–1428.
46. Mullet, W., E. P. Lai, and J. M. Yeung. 1998. Immunoassay of fumonisins by a surface plasmon resonance biosensor. *Anal. Biochem.* 258:161–167.
47. Narang, U., G. P. Anderson, F. S. Ligler, and J. Burans. 1997. Fiber optic–based biosensor for ricin. *Biosens. Bioelectron.* 12:937–945.
48. Notermans, S., and J. Nagel. 1989. Assays for botulinum and tetanus toxins, p. 319–331. *In* L. L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, San Diego, Calif.
49. Nustad, K., E. Paus, and O. P. Bömer. 1991. Magnetic particles as solid phase in immunoassays, p. 39–43. *In* J. T. Kemshead (ed.), Magnetic separation techniques applied to cellular and molecular biology. Wordsmiths' Conference Publications, Somerset, UK.
50. Ogert, R. A., J. E. Brown, B. R. Singh, L. C. Shriver-Lake, and F. S. Ligler. 1992. Detection of *Clostridium botulinum* toxin A using a fiber optic biosensor. *Anal. Biochem.* 205:306–312.
51. Olsvik, Ø., T. Popovic, E. Skjerve, K. S. Cudjoe, E. Hornes, J. Ugelstad, and M. Uhlen. 1994. Magnetic separation techniques in diagnostic microbiology. *Clin. Microbiol. Rev.* 7:43–54.
52. Poli, M. A., V. R. Rivera, and D. Neal. 2002. Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes E and F. *Toxicon* 40:797–802.
53. Potter, M., J. Meng, and P. Kimsey. 1993. An ELISA for detection of botulinal toxin types A, B, and E in inoculated food samples. *J. Food Prot.* 56:856–861.
54. Rocke, T. E., S. R. Smith, and S. W. Nashold. 1998. Preliminary evaluation of a simple in vitro test for the diagnosis of type C botulism in wild birds. *J. Wildl. Dis.* 34:744–751.
55. Roman, M. G., J. Y. Humber, P. A. Hall, R. N. Reddy, H. M. Solomon, X. M. Triscott, A. G. Beard, J. D. Bottoms, T. Cheng, and G. J. Doellgast. 1994. Amplified immunoassay ELISA-ELCA for measuring *Clostridium botulinum* type E neurotoxin in fish fillets. *J. Food Prot.* 57:985–990.
56. Rowe-Taitt, C. A., J. P. Golden, M. J. Feldstein, J. J. Cras, K. E. Hoffman, and F. S. Ligler. 2000. Array biosensor for the detection of biohazards. *Biosens. Bioelectron.* 14:785–794.
57. Sagane, Y., T. Watanabe, H. Kouguchi, H. Sunagawa, S. Obata, K. Oguma, and T. Ohyama. 2002. Spontaneous nicking in the nontoxic-nonhemagglutinin component of the *Clostridium botulinum* toxin complex. *Biochem. Biophys. Res. Commun.* 292:434–440.
58. Sakaguchi, G. 1983. *Clostridium botulinum* toxins. *Pharmacol. Ther.* 19:165–194.
59. Sakaguchi, G., S. Sakaguchi, S. Kozaki, S. Sugii, and I. Ohishi. 1974. Cross-reaction in reversed passive hemagglutination between *Clostridium botulinum* type A and B toxins and its avoidance by the

use of anti-toxic component isolated by affinity chromatography. *Jpn. J. Med. Sci. Biol.* 27:161–172.
60. Sano, M., and N. Tatsumi. 1996. Electrochemiluminescence immunoassay. *Rinsho Byori* 44:1076–1079.
61. Schantz, E. J., and L. Spero. 1967. Molecular size of *Clostridium botulinum* toxins, p. 296–301. *In* M. Ingram and A. Roberts (ed.), Botulism. Chapman & Hall, London.
62. Schiavo, G., M. Matteoli, and C. Montecucco. 2000. Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* 80:717–766.
63. Schiavo, G., C. C. Shone, O. Rossetto, F. C. Alexander, and C. Montecucco. 1993. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J. Biol. Chem.* 268:11516–11519.
64. Schmidt, J. J., and R. G. Stafford. 2002. A high-affinity competitive inhibitor of type A botulinum neurotoxin protease activity. *FEBS Lett.* 532:423–426.
65. Schmidt, J. J., R. G. Stafford, and C. B. Millard. 2001. High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. *Anal. Biochem.* 296:130–137.
66. Sharma, S. K., B. Eblen, R. L. Bull, D. Burr, and R. C. Whiting. 2003. Evaluation of hand-held *Clostridium botulinum* neurotoxin detection kits for food analysis. Presented at the first American Society for Microbiology Biodefense Research Meeting, Baltimore, 9 to 12 March 2003. Available at: http://www.asmbiodefense.org/2003tueabs.asp#185. Accessed 24 January 2005.
67. Sharma, S. K., and B. R. Singh. 1997. Hemagglutinin binding mediated protection of botulinum neurotoxin from proteolysis. *J. Nat. Toxin* 7:239–253.
68. Sharma, S. K., and B. R. Singh. 2000. Hn-33 enhances the endopeptidase activity of botulinum neurotoxin A and E against brain synaptosomal SNAP-25. *FASEB J.* 940:A1475.
69. Sharma, S. K., and B. R. Singh. 2004. Enhancement of the endopeptidase activity of purified botulinum neurotoxins A and E by an isolated component of the native neurotoxin associated proteins. *Biochemistry* 43:4791–4798.
70. Sheridan, R. E., S. S. Deshpande, and T. Smith. 1999. Comparison of in vivo and in vitro mouse bioassays for botulinum toxin antagonists. *J. Appl. Toxicol.* 1:S29–S33.
71. Shone, C., N. Appleton, P. Wilton-Smith, P. Hambleton, N. Modi, S. Gately, and J. Melling. 1986. In vitro assays for botulinum toxins and antitoxins. *Dev. Biol. Stand.* 64:141–145.
72. Shone, C. C., P. Wilton-Smith, N. Appleton, P. Hambleton, N. K. Modi, S. Gatley, and J. Melling. 1985. Monoclonal antibody–based immunoassay for type A *Clostridium botulinum* neurotoxin is comparable to the mouse bioassay. *Appl. Environ. Microbiol.* 50:63–67.
73. Shriver-Lake, L. C., R. A. Ogert, and F. S. Ligler. 1993. A fiber-optic evanescent-wave immunosensor for large molecules. *Sensors Actuators* B11:239–243.
74. Simpson, L. L. 1986. Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu. Rev. Pharmacol. Toxicol.* 26:427–453.
75. Singer, J. M., and C. M. Plotz. 1956. The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *Am. J. Med.* 21:888.
76. Singh, B. R. 2000. Intimate details of the most poisonous poison. *Nat. Struct. Biol.* 7:617–619.
77. Singh, B. R., B. Li, and D. Read. 1995. Botulinum versus tetanus neurotoxins: why is botulinum neurotoxin a food poison but not tetanus? *Toxicon* 33:1541–1547.
78. Singh, B. R., T. Lopes, and M. A. Silva. 1996. Immunochemical characterization of type A botulinum neurotoxin in its purified and complexed forms. *Toxicon* 34:267–275.
79. Singh, B. R., and M. A. Silvia. 1996. Detection of botulinum neurotoxins using optical fiber–based biosensor, p. 499–508. *In* B. R. Singh and A. Tu (ed.), Natural toxins II: structure, mechanism of action and detection. Plenum Press, New York.
80. Suen, J. C., C. L. Hatheyway, A. G. Sterigerwalt, and D. J. Brenner. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* and *Clostridium hastiforme*. *Int. J. Syst. Bacteriol.* 38:375–385.
81. Sugii, S., I. Ohishi, and G. Sakaguchi. 1977. Correlation between oral toxicity and in vitro stability of *Clostridium botulinum* type A and B toxins of different molecular sizes. *Infect. Immun.* 16:910–914.
82. Sugii, S., and G. Sakaguchi. 1977. Botulogenic properties of vegetables with special reference to the molecular size of the toxin in them. *J. Food Saf.* 1:53–65.
83. Szilagyi, M., V. R. Rivera, D. Neal, G. A. Merrill, and M. A. Poli. 2000. Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes A and B. *Toxicon* 38:381–389.
84. Tsuzuki, K., N. Yokosawa, B. Syuto, I. Ohishi, N. Fujii, K. Kimura, and K. Oguma. 1988. Establishment of a monoclonal antibody recognizing an antigenic site to *Clostridium botulinum* type B, C1, D and E toxins and tetanus toxin. *Infect. Immun.* 56:898–902.
85. van Baar, B. L., A. G. Hulst, A. L. de Jong, and E. R. Wils. 2002. Characterization of botulinum toxins type A and B by matrix-assisted laser desorption ionization and electro spray mass spectrometry. *J. Chromatogr A* 970:95–115.
86. van Baar, B. L., A. G. Hulst, A. L. de Jong, and E. R. Wils. 2004. Characterization of botulinum toxins type C, D, E, and F by matrix-assisted laser desorption ionization and electro spray mass spectrometry. *J. Chromatogr. A* 1035:97–114.
87. Wadkins, R. M., J. P. Golden, L. M. Ristiolas, and F. S. Ligler. 1998. Detection of multiple toxic agents using a planar array immunosensor ricin. *Biosens. Bioelectron.* 13:407–415.
88. Wictome, M., K. Newton, K. Jameson, B. Hallis, P. Dunnigan, E. Mackay, S. Clarke, R. Taylor, J. Gaze, K. Foster, and C. Shone. 1999. Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay. *Appl. Environ. Microbiol.* 65:3787–3792.
89. Wolfbeis, O. S. 1987. Fiber-optic sensors in biomedical sciences. *Pure Appl. Chem.* 59:663–672.
90. Yang, H., J. K. Leland, D. Yost, and R. J. Massey. 1994. Electrochemiluminescence: a new diagnostic and research tool. *Bio/Technology* 12:193.
91. Yu, H. 1998. Comparative studies of magnetic particle-based solid phase fluorogenic and electrochemiluminescent immunoassay. *J. Immunol. Methods* 218:1–8.
92. Yu, H., and J. G. Bruno. 1996. Immunomagnetic-electrochemiluminescent detection of *Escherichia coli* O157 and *Salmonella typhimurium* in foods and environmental water samples. *Appl. Environ. Microbiol.* 62:587–592.
93. Yu, H., and J. G. Bruno. 1996. Development of an immunomagnetic assay system for rapid detection of bacteria and leukocytes in body fluids. *Appl. Environ. Microbiol.* 629:3474–3476.
94. Zhang, Z., and B. R. Singh. 1995. A novel complex of type E *Clostridium botulinum. Protein Sci.* 4(Suppl. 2):110.