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Technologies for Detecting Botulinum Neurotoxins in Biological and Environmental Matrices

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Abstract

Biomonitoring of food and environmental matrices is critical for the rapid and sensitive diagnosis, treatment, and prevention of diseases caused by toxins. The U.S. Centers for Disease Control and Prevention (CDC) has noted that toxins from bacteria, fungi, algae, and plants present an ongoing public health threat, especially since some of these toxins could compromise security of the food supply. Botulinum neurotoxins (BoNTs), produced by *Clostridium* spp., are among those bacterial toxins that pose life-threatening danger to humans. BoNTs inhibit the release of acetylcholine at peripheral cholinergic nerve terminals and cause flaccid paralysis. BoNTs are grouped in seven serotypes and many subtypes within these groups. Rapid and accurate identification of these toxins in contaminated food as well as in environmental matrices can help direct treatment. Herein, we discuss current methods to detect BoNTs with a focus on how these technologies have been used to identify toxins in various food and environmental matrices. We also discuss the emergence of new serotypes and subtypes of BoNTs and the increasing number of cases of botulism in wildlife. Finally, we consider how environmental changes impact food safety for humans and present new challenges for detection technology.

Keywords: Botulism, Toxins, Food matrix, Environmental detection, Foodborne illness

1. Introduction

The U.S. Centers for Disease Control (CDC) have summarized the risks that biological toxins pose to human health [1]. Bacteria, fungi, parasites, and plants all produce toxins in the environment that can impact food safety. Furthermore, changes in the environment have caused emergence of new problems associated with toxins. One example is the production of toxins by *Clostridium botulinum*. This pathogen, which is a gram-positive, anaerobic spore-

forming bacterium, produces botulinum neurotoxins (BoNTs). Humans are susceptible to the effects of these poisons, which are among the most toxic molecules known [1]. The parenteral lethal dosage for humans is 0.1–1 ng/kg, and the oral dose is 1 µg/kg. A single gram of BoNT released into the environment and subsequently inhaled could kill more than one million people [2, 3]. BoNTs exert their biological effects by blocking acetylcholine release by neurons. To date, BoNTs have been divided into seven serotypes, denoted as A through G, of which A, B, E, and F are known to be toxic to humans [4–6]. However, all of the botulinum serotypes are possibly toxic to people. In addition to the principal serotypes, at least 40 additional subtypes have been described based on differences in both primary peptide sequence and three-dimensional structure [4–6]. In this discussion, we review the basic biology of BoNTs and current methods to detect these molecules in biological and environmental matrices.

C. botulinum isolates are categorized into different groups [5, 6]. Members of Group I are referred to as “proteolytic” and produce toxin types A, B, or F. They are widely distributed in the environment and often found in various raw foods. BoNTs can cause symptoms at levels as low as 5 ng. Although the onset of symptoms typically takes 12–36 hours, the time course depends on the amount of toxin ingested [2, 3]. It can take much longer for symptoms to manifest. Initial symptoms include diarrhea and vomiting followed by neurological effects that include blurred vision, weakness, and difficulty in swallowing, talking, and breathing. Unless diagnosed early, mortality rates can be as high as 40% [1–4]. Timely response and current treatments have reduced mortality to less than 10%. The most common foods involved in outbreaks are improperly preserved meat or fish products, but a range of other foods have been implicated, such as cheeses (including vegetables preserved in oil and cheese). Because botulinum toxins are not heat stable, they can be inactivated at cooking temperatures.

Strains in Group II are classified as “non-proteolytic” [5, 6]. These *C. botulinum* strains synthesize neurotoxin B, E, or F. These bacteria can grow at temperatures <3°C are ubiquitous in the environment. Moreover, one can find Type E strains in aquatic habitats [1–4]. It is not known whether these strains can synthesize neurotoxins in refrigerated processed foods without visible spoilage. The endospores of strains in this group are not as resistant to heat as those strains in Group I. Neurotoxins synthesized by strains in Group II toxins have shown to be less potent than those of Group I; at least 0.1 µg of neurotoxin is required to cause symptoms of botulism. However, their other biological properties are similar. Foods involved in outbreaks of Group II botulism include cold-smoked fish and other preserved fish products.

Group III botulinum produces toxins of serotype C or D and is associated with avian and non-human mammalian botulism [5, 6]. Whole genome sequencing analysis indicates that strains of physiological group III are probably more related to *Clostridium haemolyticum* and *Clostridium novyi* than to *C. botulinum* serotypes belonging to Groups I and II. Group IV is rare and has not been well characterized. However, it does synthesize neurotoxin serotype G.

Bacteriophages contain the neurotoxin genes of *C. botulinum* serotypes C and D [5, 6]. The BoNT prophage replicates in the bacterium as a large plasmid, and strains containing the phage can become toxigenic via either type C or type D phages. The distinction between types C and D is not clear because chimeric sequences have been isolated from the environment. These toxin genes have been identified in avian isolates. They contain sections from both BoNT/C

and BoNT/D genes and are referred to as type C/D [5, 6]. The chimeric toxin is more pathogenic to avians than either serotype C or D individually. In *C. botulinum* serotypes C and D, there is a small amount of a binary toxin, denoted as the C2 toxin. The genes encoding the C2 toxin have been localized to a plasmid. Structurally and functionally, the C2 toxin contains a translocation domain and an ADP-ribosylating domain that has been shown to target cellular actin. The occurrence of other chimeric botulinum toxin genes has yet to be determined [5, 6].

At the amino acid sequence level, BoNT serotypes can differ from one other by 34–64% [5, 6]. Significant genetic variation within each serotype has also been observed. In fact, 32 toxin subtypes with amino acid sequence differences of 2.6–32% have been identified thus far, and more will likely be identified in the future [5, 6]. This serotype and subtype diversity confound direct antibody and molecular-based assay designs. It is rare that one probe can bind to all serotypes. In *C. botulinum*, the neurotoxins are first synthesized as a large holotoxin (approximately 150 kDa). They are then processed by a trypsin-like protease in *C. botulinum* yielding two polypeptides (one approximately 100 kDa and the other approximately 50 kDa) that are still bound by a single disulfide [2, 3]. The neurotoxin structure mimics other known A–B dimeric toxins found in other bacterial pathogens. The ~100 kDa fragment is called the heavy chain (HC) and aids the binding of the neurotoxin to host cell receptors and its translocation from vesicles to the cytoplasm [2, 3]. The ~50 kDa fragment, called the light chain (LC), contains the enzymatically active domain of the neurotoxin. Recombinantly, expressed LC is routinely used for activity-based neurotoxin assays. Antibodies specific for the HC and LC are used for immunoassays for detecting neurotoxins as well as for neutralization.

2. Important factors to consider when developing toxin detection assays

The development of a robust assay for the detection of any pathogen or biological product of a pathogen (such as a toxin) requires consideration of several factors: sensitivity, specificity, matrix effects, and biological activity [8–10]. Each of these factors is briefly discussed below in the context of assay methods for *C. botulinum* toxins.

3. The mouse bioassay

In the laboratory, a rodent bioassay is considered the “gold standard” method for detecting BoNTs [8–10]. Despite much effort to replace the use of animals, it is still the most sensitive and reliable assay to model all aspects of BoNT intoxication: binding, translocation, enzymatic activity, and pathology. Alternatives to the mouse bioassay have been developed (discussed below) with shorter assay times and equal or greater sensitivity.

The mouse assay quantitatively determines the amount of BoNT required to kill all mice in a test group. This measurement is expressed as a minimal lethal dose (MLD). Although protocols may vary, mice are usually injected intraperitoneally with 0.5 mL of BoNT sample in a dilution series and then monitored over several days for signs of intoxication and death

[11, 12]. If enough sample is available for an assay, the specific neurotoxin can be identified using neutralization with antibodies specific to each of the neurotoxin serotypes (A–G). The toxin serotype is therefore revealed based on which antibody confers protection from death. The mouse bioassay is highly sensitive and useful for detection of different neurotoxins in different matrices. However, despite its versatility, the mouse assay has limitations that include: long assay times and the use of animals requiring specialized animal facilities, substantial costs, trained staff, and consideration of ethical issues, especially when death is used as an endpoint. There is also substantial variation in results observed among different research laboratories [8–12].

Alternative “refined” animal assays that do not use death as an endpoint, such as the mouse phrenic nerve hemi-diaphragm assay, have been evaluated [13, 14]. Despite being more sensitive and rapid compared to the use of whole animals, these assays usually require the use of specific equipment and personnel with specialized training. Furthermore, these alternative animal assays are not feasible with larger samples and those containing a complex matrix. However, a recent study described an *in vivo* assay using a toe-spread reflex model. This method was used to detect neurotoxins in simple buffer solutions, samples containing serum, and milk [15]. This new assay provides results more quickly than standard mouse bioassays. Whether these results can be translated into a user-friendly, deployable kit has yet to be determined.

4. DNA and other nucleic acid–based methods

Numerous nucleic acid methods have been developed for detecting clostridial DNA in biological and environmental matrices. The polymerase chain reaction (PCR) to identify the presence of *C. botulinum* DNA was originally used to detect the presence of bacterial spores in samples [16]. The method is capable of detecting the presence of as few as 100 spores per reaction mixture for serotypes A, E, and F and only 10 spores per reaction mixture for serotype B.

Multiplex PCR methods have also been developed to analyze unknowns for a battery of different targets such as different pathogens and/or associated gene products of these pathogens. Multiplexed assays employ different combinations or sets of PCR primers, each one specific for a gene of interest, to amplify multiple targets in one PCR tube. One such multiplex method could possibly discriminate among BoNT serotypes A, B, E, and F. As previously described, Peck et al. [16] developed a culture enrichment method that, when coupled with multiplex PCR, could identify strains of *C. botulinum* that were non-proteolytic (such as BoNT serotypes B, E, and F). This method was robust and rapid enough for use with food samples contaminated with *C. botulinum*.

Real-time PCR (RT-PCR) or quantitative PCR (qPCR) is also useful in studies of gene expression, specifically differential expression of genes under various environmental conditions or comparative studies of different organisms. For detection of clostridial DNA, RT-PCR methods examine expression of the NTNH (non-toxic, non-hemagglutinin) and numerous other genes in *C. botulinum* serotypes A, B, E, and F [18]. Pentaplex methods have been developed to

simultaneously identify and discriminate among larger numbers of different serotypes, using a wider array of different genes [19]. This technology may prove to be efficient and cost-effective.

The GeneDisc Cyclor is an apparatus to perform RT-PCR applications using the GeneDisc system. The GeneDisc is a disposable plastic reaction tray that is the size of a compact disc. This method has been designed for simultaneously testing for the BoNT/A, BoNT/B, BoNT/E, and BoNT/F genes. In 2011, Fach et al. evaluated the GeneDisc Cyclor equipment with neurotoxin-producing clostridia and non-BoNT-producing bacteria isolated from various clinical, food, and environmental samples [20]. Results obtained using this “macroarray” were also compared to the mouse bioassay. The toxin genes were detected in all clostridial serotypes A, B, E, and F as well as in toxigenic *Clostridium baratii* Type F and toxigenic *Clostridium butyricum* Type E. No cross-reactivity was observed with bacteria not toxigenic to humans as well as *C. botulinum* Types C, D, and G. An evaluation of the GeneDisc array was performed in four European laboratories with BoNT-producing clostridia and 10 different samples that included food matrices and clinical isolates [20]. Results demonstrated the technology to be specific and reliable in the identification of *C. botulinum* cells containing genes encoding neurotoxins A, B, E, and F. Furthermore, contaminated food and fecal samples were successfully tested. This assay is highly sensitive, capable of detecting as low as 5–50 genome copies in each PCR assay. The GeneDisc Cyclor can also be used for monitoring neurotoxin-producing clostridia in food samples, clinical samples, and environmental matrices. A similar study was carried out examining a focused microarray for detection of genes-encoding BoNTs [21].

Recently, Kolesnikov et al. [22] described a new method called “proteolytic PCR” in which PCR is used to assay the proteolytic activity of botulinum toxin. This technology starts with DNA–protein complexes attached to a solid phase. Proteolytic cleavage releases DNA into solution. The DNA can then serve as a template for PCR. This study described its use to detect botulinum toxin and tetanus toxin proteolytic activity [22].

5. Immunological and antibody-based assays

Enzyme-linked immunosorbent assay (ELISA) is a common assay used to detect BoNTs. This method utilizes anti-BoNT capture and detector antibodies arranged in a “sandwich” format. The detection formats are most commonly luminescent- or colorimetric-based. Prior generations of BoNT immunoassays were approximately 10 times less sensitive than the mouse bioassay described in the previous section. Although not as sensitive, ELISA methods are relatively fast, inexpensive, and simple to perform. They are also less subject to inhibitory matrix effects. An amplified ELISA for detecting toxins in food matrices has also been described [23]. Toxins for serotypes A, B, E, and F could be detected in liquids, solid food, and semisolid food. Assay performance was evaluated in a range of food matrices, such as broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meats, and dairy foods. The assay sensitivity varied for each botulinum serotype. The tests readily detected 2 ng/mL of serotypes A, B, E, and F in various foods tested. Recently, traditionally

formatted, very sensitive sandwich ELISAs used high affinity mAbs against BoNT/A and BoNT/B to detect BoNT/A as low as 5 and 25 pg/mL in buffer and in a milk matrix, respectively; and BoNT/B at 100 fg/mL and 39 pg/mL in buffer and milk matrix, respectively [24–26].

These mAbs were used in an electrochemiluminescence (ECL) immunosorbent assay using the Sector 2400 Imager (Meso Scale Discovery [MSD], Rockville, MD, USA) instrument [27, 28]. Detection sensitivities for BoNT/A in this system were similar to traditional ELISAs in buffer but were markedly improved in liquid food matrices because of the reduced background signal. The higher sensitivity and reduced time required for these new immunosorbent methods make them potential alternatives to the mouse bioassay. Sharma et al. recently developed another ECL assay for simultaneous detection of several biothreat agents (including clostridial neurotoxins) in milk products, with limit of detection (LOD) of 40 pg/mL for BoNT/A complex [28]. The ECL assay was also successfully applied to screen *C. botulinum* serotype A outbreak strains. The study also showed that this sensitive ECL assay is rapid (it can be completed in less than 6 hours). The ECL assay also has potential for using as an *in vitro* screening method, complementing or replacing other immunoassays.

Cheng and Stanker [27] evaluated the performance of antitoxin mAbs using the same electrochemiluminescence immunoassay platform (Sector 2400 Imager, MSD). The ELISA and ECL methods were observed to be more sensitive than the mouse bioassay. In fact, the ECL assay was able to outperform ELISA in terms of detection sensitivity—including food matrices spiked with BoNT/A and in some food matrices spiked with BoNT/B. The ELISA and ECL methods are fast and simple alternatives to the mouse bioassay and can be used for detecting BoNTs in food matrices and serum samples.

One example of mAb development using a recombinant immunogen was the work of Liu et al. [29], who expressed the recombinant H(C) subunit of BoNT type A (rAH(C)). Two out of 56 mAbs were selected to establish a highly sensitive sandwich chemiluminescence enzyme immunoassay (CLEIA) with LOD for both rAH(C) and BoNT/A of 0.45 pg/mL. This CLEIA can be used to detect BoNT/A in matrices, such as milk and beef extract. This method is 20–40-fold more sensitive than the mouse bioassay and takes only 3 hours to complete, making it a useful method to detect and quantify BoNT/A.

The multiplex technology discussed above to detect nucleic acid has also been applied to the development of methods to analyze multiple epitopes on a single antigen and multiple targets in a single sample. This approach uses multiple mAbs as well as polyclonal antibodies to reduce false-positive and false-negative results. A commercialized system, Luminex xMAP technology, utilizes microsphere beads conjugated with antibodies. It employs paramagnetic beads instead of non-magnetic polystyrene beads and is very useful for the analysis of food matrices. The antibody-bead complexes detect multiple epitopes in a single sample. This technology was used to detect abrin, ricin, BoNTs, and staphylococcal enterotoxins in spiked food samples [30].

Zhang et al. [31] developed ELISA-based protein antibody microarrays to simultaneously detect six serotypes of BoNTs. Using numerous different food and other matrices, the microarray is capable of detecting BoNT serotypes A through F. Using engineered, high-affinity

antibodies, these serotypes were detected to similar levels in various matrices and were comparable to detection in buffer.

Accurate and sensitive detection of contaminated food and other biological samples in the environment is critical. Brunt et al. [32] have developed an affinity column-based assay for detecting neurotoxin in food matrices—specifically serotypes A, B, E, and F. The detection limit for BoNT/A was reported as 0.5 ng, which is two-fold more sensitive than lateral flow methods (also see Section 6) [32]. For serotypes B, E, and F, the minimum detection limit ranged from 5 to 50 ng. Although not as sensitive as ELISA or mouse bioassay, rapid immunochromatographic methods generally require only 15–30 minutes to complete. They do not require enrichment steps and are amenable to use in the field.

Koh et al. have presented a new technology called SpinDx [33]. This method utilizes a centrifugal microfluidic platform to detect BoNTs based on a sedimentation immunoassay. A reagent mixture is prepared, consisting of capture beads conjugated with target-specific antibodies and fluorescent detection antibodies. The reagents are mixed with the sample and forced through a channel containing dense medium, a process that washes the sample and removes interfering substances. The beads that collect at the end of the channel are queried to determine the amount of antigen bound. SpinDx was used to quantify BoNTs with sensitivity that surpassed the mouse assay.

6. Lateral flow methods

The development of lateral flow methods for detecting toxins has also led to the commercial availability of numerous kits for sensitive and rapid testing. Lateral flow methods employ capture antibodies that are “printed” on nitrocellulose membranes in a process akin to inkjet printing technology. Detection antibodies are labeled with visible materials, such as colloidal gold or colored latex beads. The sample is added to a reagent pad containing labeled toxin-binding detection antibodies and is wicked across the membrane. Toxin is retained by the capture antibody, which also concentrates the labeled detection antibody. A positive reaction is revealed as a colorimetric change and is presented as a line on the device. In general, lateral flow methods are qualitative and simply determine the presence or absence of neurotoxin. Sharma et al. [34] compared several commercial lateral flow devices for their capacities to detect toxin in food samples. They were able to detect BoNT/A and BoNT/B as low as 10 ng/mL and BoNT/E at 20 ng/mL in various liquids, such as milk, soft drinks, and fruit juices. Ching et al. [35] used the same mAbs described in the ELISA section above [24–26] in lateral flow devices to achieve sensitivities of 0.5 and 1 ng/mL for BoNT/A in buffer and milk, respectively. Although simple lateral flow tests have lower sensitivities compared to other methods, they produce rapid results and are most useful for the rapid screening of samples suspected of frequent contamination at relatively high level of BoNT. They have many applications and are ideal for field use by non-technical personnel. Self-contained and not necessarily requiring additional reagents or equipment, they can be easily interpreted in the field.

An innovative approach for toxin detection has recently been developed that combines antibodies with the amplification power of PCR, immuno-PCR (I-PCR) [36]. In I-PCR, template DNA is conjugated to the antibody, replacing a secondary antibody conjugated to the detection enzyme. Upon binding of toxin by the antibody, the presence of toxin is revealed using PCR. Chao et al. [36] described an I-PCR method for detection of BoNT/A with femtogram (10^{-15} g) sensitivity. These investigators compared competitive and sandwich ELISA to the I-PCR method. The I-PCR method was 10^3 – 10^5 times more sensitive with LODs for the ELISA methods of about 50 fg. The use of I-PCR for highly sensitive detection of BoNT in food matrices or other biological and environmental backgrounds has yet to be reported (as of late 2015).

7. Mass spectrometry-based methods to detect toxins

Mass spectrometry (MS) has been used as a method to dissect components of botulinum toxin complexes [37–39]. The MS-based method, called ENDOPEP-MS, uses antibodies to concentrate and extract BoNT from test samples [38]. The concentrated toxins are then subjected to an endopeptidase activity-based assay to generate target cleavage products. Finally, MS is used to identify these products. This approach has been successful in identifying BoNT serotypes A, B, E, and F in various food and clinical matrices with greater sensitivity than the mouse bioassay.

Morineaux et al. [40] recently described a MS method that employs immunocapture enrichment by antibodies specific for BoNT/A-L chains. The enriched analyte is then analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) on a triple quadrupole mass spectrometer (QqQ) in multiple reaction monitoring (MRM) mode. Peptides from BoNT LC specific to the subtypes BoNT/A1–A3 and BoNT/A5–A8 could be identified. BoNT/A subtypes were correctly identified in culture supernatants, water, and orange juice samples with a LOD of 20–150 mouse lethal doses (LDs), but there was a lower sensitivity in serum samples.

Kalb et al. [41] have described the development of a quantitative enzymatic method for the detection of four BoNT serotypes using matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) MS. Factors that might affect the linearity and dynamic range for detection of BoNT cleavage products were carefully examined, including the concentration of the substrate and internal standard, the length of time for the cleavage reaction, and the components present in the reaction solution. Longer incubation time produced more sensitive results but was not capable of determining higher toxin concentrations, whereas a shorter incubation time was less sensitive. To address these limitations, a novel two-step analysis was developed [41]. By combining the results from a two-stage quantification, four or five orders of magnitude in dynamic range are observed for detection of BoNT serotypes A, B, D, and F. To minimize the number of cleavage reactions and analytical samples, the assay can be multiplexed using mixtures of different neurotoxin substrates. Numerous different research groups (including Kalb et al. [42], Björnstad et al. [43], and Hines et al. [37]) have used MS to dissect the components of the BoNT/G complex, revealing BoNT/G as well as other toxin protein components, namely NTNH, HA-17, HA-33, and HA-70. Overall, the use of MS can provide rapid and definitive results.

8. Enzymatic assays to detect toxins

Rapidly distinguishing between the presence of active versus inactive toxin is critical for effective medical intervention in toxicoses. As BoNTs are zinc metalloproteases, knowledge of the human targets for these enzymes has enabled development of enzyme-substrate assays. Activity assays have been developed using a wide variety of detection systems. Toxin samples can be treated with recombinant versions of host–target substrates (such as SNAP-25), and the cleavage products can be detected using immunoblotting. Alternatively, fluorogenic peptide substrates emit a signal when cleaved. One such system uses a peptide (“SNAPTide”) with reverse-phase HPLC and a fluorescence detector to detect as low as 5 pg/mL of BoNT/A in skim milk [45]. Other peptide substrates (VAMPtide and SYNTAXtide) have been used for detection of their cognate BoNTs [46]. The levels of substrate cleavage correlate well with toxin activity.

9. Cell-based assays

Cell-based assays measure BoNT receptor binding, translocation, and enzymatic activity and can be *in vitro* alternatives to the mouse bioassay. Several neuronal and non-neuronal cell lines have been analyzed for use in neurotoxin assays. These include the following: BE(2)-M17 cells, chick embryo neuronal cells, neuroblastoma cells, and rat spinal cord cells [47–50]. In general, the endpoint of cell-based assays for BoNT/A is the proteolytic cleavage of its intracellular substrate, the vesicle-trafficking SNARE protein called SNAP-25. Recently, Hubbard et al. [51, 52] described the functional analysis of numerous different biological neurotoxins, including BoNTs, in networked cultures of stem cell–derived central nervous system neurons. The investigators demonstrate synaptic activity in cultured neurons of humans and rodents, suggesting that these could serve as comparable methods to animal studies. Hong et al. [53] have also developed a similar assay using a motor neuron-like continuous cell line. Pathe-Neuschäfer-Rube et al. [54] developed a N-terminal tagged luciferase-expressing neuronal cell line. Luciferase is released from these transfected cells during depolarization, which is blocked by botulinum toxin. Cell-based methods may prove to be equally sensitive, or better, than animal studies and may provide a new alternative for *in vivo* experiments. For example, for the first time, the U.S. Food and Drug Administration approved a cell-based assay developed by the biotechnology company Allergan, Inc. (Irvine, CA, USA) for its use as an alternative to the mouse bioassay. However, the details of the Allergan assay have not been published.

10. New antibody and biosensor technologies

Diamant et al. [55] have used an interesting approach for generating antibodies that have higher specificity against serotypes A, B, and E, and possess neutralizing capabilities. Mice were immunized with a “trivalent mixture” of recombinant fragments of neurotoxins A, B,

and E. The method generated numerous different monoclonal antibodies against each serotype. Most of the monoclonal antibodies had higher ELISA titers compared to polyclonal antibodies and had specificities with five orders of magnitude greater specificity. These antibodies also protected against neurotoxin dosages of 10–50 LD₅₀. They also observed a neutralizing synergy when serotype-specific monoclonal antibodies were combined into an oligoclonal mixture.

Detection methods can also utilize highly sensitive antibodies to enrich or enhance sample preparation as well as amplify the signal. For example, an assay with a large immunosorbent surface area (ALISSA) [56, 57] utilizes an antibody to concentrate the neurotoxin onto the surface of a large bead. The “captured” toxin molecules are then used in an enzyme assay. Using food matrices, the LOD for ALISSA was observed as low as 50 fg/mL. This is far more sensitive than the mouse bioassay, immunoassay, or enzyme assay and suggests that it may be useful for detecting food contamination. Marconi et al. [58, 59] have also described the use of surface plasmon resonance (SPR) to examine synaptic vesicle capture by antibodies against BoNT substrates, such as SNAP25 and VAMP2. SPR could be used with cultured neurons in 96-well plates incubated with either BoNT/A or BoNT/B and may be an alternative to animal studies. Further development of label-free and optical biosensors for detecting botulinum toxin [61, 62] will provide additional technologies with possible impact on food safety.

11. Challenges for botulinum neurotoxin detection: new serotypes in the environment

Kull et al. [62] described the isolation of a novel *C. botulinum* strain associated with an outbreak of botulism in Germany. Genotyping of the isolate and subsequent comparison of its neurotoxin gene sequences with database sequences revealed it as a novel BoNT/A serotype. This novel isolate has been called BoNT/A8, and its neurotoxin gene is located within a HA-orfX+ locus. Unique among all other BoNT/A subtypes known so far, an arginine insertion was identified in the HC domain of the HC. Both the full-length neurotoxin and the recombinant LC of BoNT/A8 had lower endopeptidase activity compared to BoNT/A1. Reduced ganglioside binding and lower enzymatic activity may both contribute to lower biological activity of BoNT/A8 as determined using the phrenic nerve hemi-diaphragm assay. Nevertheless, the novel BoNT/A8 subtype caused severe botulism in a 63-year-old male. These findings reiterate that subtyping of BoNT is highly relevant to food safety, epidemiology, and clinical diagnostic and therapeutic practices. Hill et al. [63] carried out a detailed genetic analysis of *bont* genes and confirmed their location on chromosomes, phagemids, and plasmids, as well as variations among different genes. Close examination of sequences confirmed that horizontal gene transfer, site-specific insertions, and recombination events have contributed to the observed variation among different neurotoxins. Understanding the details of toxin gene sequences, protein sequences, and their function can pave the way for the development of novel therapeutics and tailor-made antitoxins. Ongoing development of diagnostics for new and emerging toxins is critical to food safety and human health.

12. Botulinum neurotoxin detection in the environment: role of climate change and algal blooms in avian botulism and the challenges of environmental matrices

Increased global temperature has been associated with increased algal blooms. The role of these algal blooms in disease is unclear. However, recently, a connection between algal blooms and botulism has been explored. Avian botulism is a disease that often occurs on a yearly cycle and results from the ingestion of neurotoxins by birds. This disease has become increasingly common in the U.S. Great Lakes [64], as have blooms of the green alga *Cladophora*, which can serve as a potential habitat for *C. botulinum*. The interactions between *Cladophora* and *C. botulinum* are unclear due to the complex food web associated with this disease. Investigators in several recently published studies [64–66] reported a high number of botulism cases in shoreline birds in Lake Michigan. This increased incidence was correlated with increasingly large accumulations of *Cladophora* in the water. Sadowsky et al. [65] examined algal mats that were collected from Lakes Michigan, Ontario, and Erie in 2011–2012 and then compared them with algal populations in sand and water. They found that 96% of *Cladophora* mats collected from the shorelines in 2012 contained *C. botulinum* Type E. Among the algae samples containing detectable *C. botulinum*, the large number of detected *C. botulinum* type E cells indicated that *Cladophora* mats are principal sources of this pathogen. Mouse toxin and antitoxin bioassays further confirmed the toxin in collected samples as serotype E. Further examination of *Cladophora*-associated *C. botulinum* may lead to a model system to study algal–clostridial interactions and result in lower bird mortality.

In a follow-up study using PCR, Sadowsky et al. [66] reported that algae mats from different shores of the Great Lakes contained the serotype E gene. Also, *C. botulinum* was found to be present in amounts of up to 15,000 cells per gram of dried algae, based on quantitation of gene copies encoding serotype E. Moreover, genes for serotypes A and B, which are associated with human diseases, were detected in several of the algal samples. Using mouse toxin assays and subsequent neutralization assays, it was confirmed that *Cladophora*-associated *C. botulinum* was serotype E. One might consider that with increased incidence of extreme drought and other environmental changes, algal blooms may happen more often in water-restricted areas, and *C. botulinum* growth may pose a threat to humans if toxin is produced in algal mats. Developing sensitive detection methods for toxins within algal matrices is urgent, as is monitoring other matrices that could provide an environment for botulinum toxin production. The increased avian botulism associated with increased algal blooms highlights the need to develop new technologies for detection of toxin in the environment, or a re-evaluation of current methods and their use in environmental matrices.

Vidal et al. [67] examined numerous environmental factors that influence the prevalence of the unusual mosaic BoNT serotype C/D. Between 1978 and 2008, 13 avian botulism outbreaks were observed, killing 20,000 birds. A significant association was found between the number of dead birds recorded in each botulism outbreak and the mean temperature in July (with average temperatures being higher than 26°C). The presence of *C. botulinum* type C/D in wetland sediments was detected by qPCR. Furthermore, low concentrations of chloride ions

and high organic matter content were correlated with the presence of *C. botulinum*. The digestive tracts of dead birds found during botulism outbreaks were also analyzed; *C. botulinum* was present in almost 40% of the studied samples. Recently, Le Maréchal et al. [68] examined livers from dead birds suspected of having botulism and showed that this organ can serve as a reliable matrix for RT-PCR confirmation of disease. This finding may provide wildlife investigators with a faster method to confirm avian deaths due to botulism.

The presence of *C. botulinum* was detected in aquatic invertebrates and flesh-eating invertebrates collected around bird carcasses. Moreover, the presence of *C. botulinum* bacteria in the adult fly stage of some invertebrates raises the question of whether flies can transport *C. botulinum* from one carcass to another. The same investigators examined whether adult blowflies could play a significant role in botulism outbreaks by carrying *C. botulinum* between carcasses. A field experiment and subsequent laboratory tests determined that blowflies could transport *C. botulinum* Type C/D between carcasses [69]. These results confirm that adult flesh-eating flies could play a role in avian botulism outbreaks. An environmental monitoring protocol for botulinum-carrying flies has not yet been established. It is a matter for future research to determine whether these or other insects could serve as mechanical vectors for botulinum isolates that pose greater threats to humans than the avian isolates.

Probably, one of the greatest challenges is determining which environmental matrices should be collected and analyzed, and which ones would provide the most definitive information about potential threats to humans and animals. For instance, Anza et al. [70] examined the role of eutrophication and avian botulism outbreaks in wetlands receiving effluents from urban wastewater treatment plants. Numerous different avian pathogens, including clostridial pathogens, were present in wastewater and could pose a threat to birds living in wastewater wetlands. Methods to detect BoNTs in environmental matrices could be adapted from previous studies of food and clinical samples or may require new technologies. Future studies in this area are clearly warranted.

13. Future technologies to detect botulinum neurotoxins

The discussion herein has presented a general overview of methods currently being used to detect BoNTs. Many current methods to detect BoNTs in food and environmental matrices have been adapted from the clinical laboratory. New possibilities to consider, to name a few, could exploit the tools of nanotechnology, mHealth, and the use of mobile devices, the capability of miniaturization for even more sensitive and rapid detection of BoNTs. The application and practical use of these technologies might be valuable advancements to current methods to detect BoNTs.

14. Conclusions and recommendations

To maintain a safe food supply and to detect toxins in an ever-changing environment, an ongoing, concerted effort in assay development and validation is essential for human health

and safety. Some areas for investigators to consider include the development of new antibodies and binding molecules specific to BoNT serotype F as well as new hybrid serotypes. The impact of different types of neurotoxin accessory proteins on the detection of BoNTs should also be examined. Furthermore, the impact of food processing conditions on the stability and bioavailability BoNTs is an area in need of further study. The development of new bioassays based on non-mammalian systems and cell cultures should also be supported as well as the advancement of new portable and field-deployable testing methods, including those based on miniaturization of current bench top instruments. These are only a few recommendations, but their development and use should help to further ensure food safety and animal and human health.

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