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Alternative Methods for Testing Botulinum Toxin: Current Status and Future Perspectives

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Abstract

Botulinum toxins are neurotoxic modular proteins composed of a heavy chain and a light chain connected by a disulfide bond and are produced by *Clostridium botulinum*. Although lethally toxic, botulinum toxin in low doses is clinically effective in numerous medical conditions, including muscle spasticity, strabismus, hyperactive urinary bladder, excessive sweating, and migraine. Globally, several companies are now producing products containing botulinum toxin for medical and cosmetic purposes, including the reduction of facial wrinkles. To test the efficacy and toxicity of botulinum toxin, animal tests have been solely and widely used, resulting in the inevitable sacrifice of hundreds of animals. Hence, alternative methods are urgently required to replace animals in botulinum toxin testing. Here, the various alternative methods developed to test the toxicity and efficacy of botulinum toxins have been briefly reviewed and future perspectives have been detailed.

Key Words: Botulinum toxin, Acetylcholine, *In vitro*, Alternative studies

INTRODUCTION

Clostridium botulinum is an anaerobic, gram-positive, spore-forming, rod-shaped bacterium that produces neurotoxic proteins called botulinum toxins (Nigam and Nigam, 2010). Food-borne poisoning cases of botulinum toxins were first observed in eighteenth-century Europe, and the condition was termed 'sausage poisoning' or botulism as 'Botulus' means sausage in Latin (Kerner, 1817). Depending on the type of illness caused by botulinum toxins, *C. botulinum* strains are divided into four different groups. Bacterial groups I and II are associated with the human illness, group III is associated with illness in animals, and group IV is not related to any illness (Nawrocki *et al*., 2018). So far, depending on the serological properties of the toxins, at least seven different types (A-G) of botulinum toxins have been identified from different *C. botulinum* strains (Nawrocki *et al*., 2018). Botulinum toxins A, B, and F are produced by group I bacteria, and toxins B, D, and E are produced by group II bacteria (Lindström and Korkeala, 2006). Botulinum toxin types A, B, and E have been identified as the most common neurotoxins causing human poisoning, whereas toxin types C and D are rarely associated with human toxicities; type F causes minimal human toxicity (Hodowanec and Bleck, 2015). In addition to *C. botulinum*, several other strains of bacteria can produce botulinum toxins, e.g., *C. butyrricum*, *C. barati*, and *C. argentinensis* (Hodowanec and Bleck, 2015; Pirazzini *et al*., 2017).

MECHANISM OF TOXICITY

Acetylcholine is a neurotransmitter that functions at neuromuscular junctions to activate muscles. For muscles to respond, several events are crucial (Fig. 1). First, three soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, synaptobrevin, SNAP-25, and syntaxin, form a complex. Second, the synaptic vesicle and terminal membrane fuse to release acetylcholine into the synaptic cleft. Third, acetylcholine binds to the acetylcholine receptor in muscles and the muscle fibers contract (Fig. 1A). All serotypes of botulinum toxin consist of a 150-kDa, single-chain progenitor toxin, which can be triggered by a protease to produce a 100-kDa heavy chain and a 50-kDa light chain. When the toxin is internalized into nerve cells, the interchain disulfide bond is broken, releasing the light chain possessing endopeptidase activity. This light chain specifically cleaves one of the three SNARE proteins involved in neurotransmitter release (Hodowanec and Bleck, 2015; Pirazzini *et al*., 2017). This pre-

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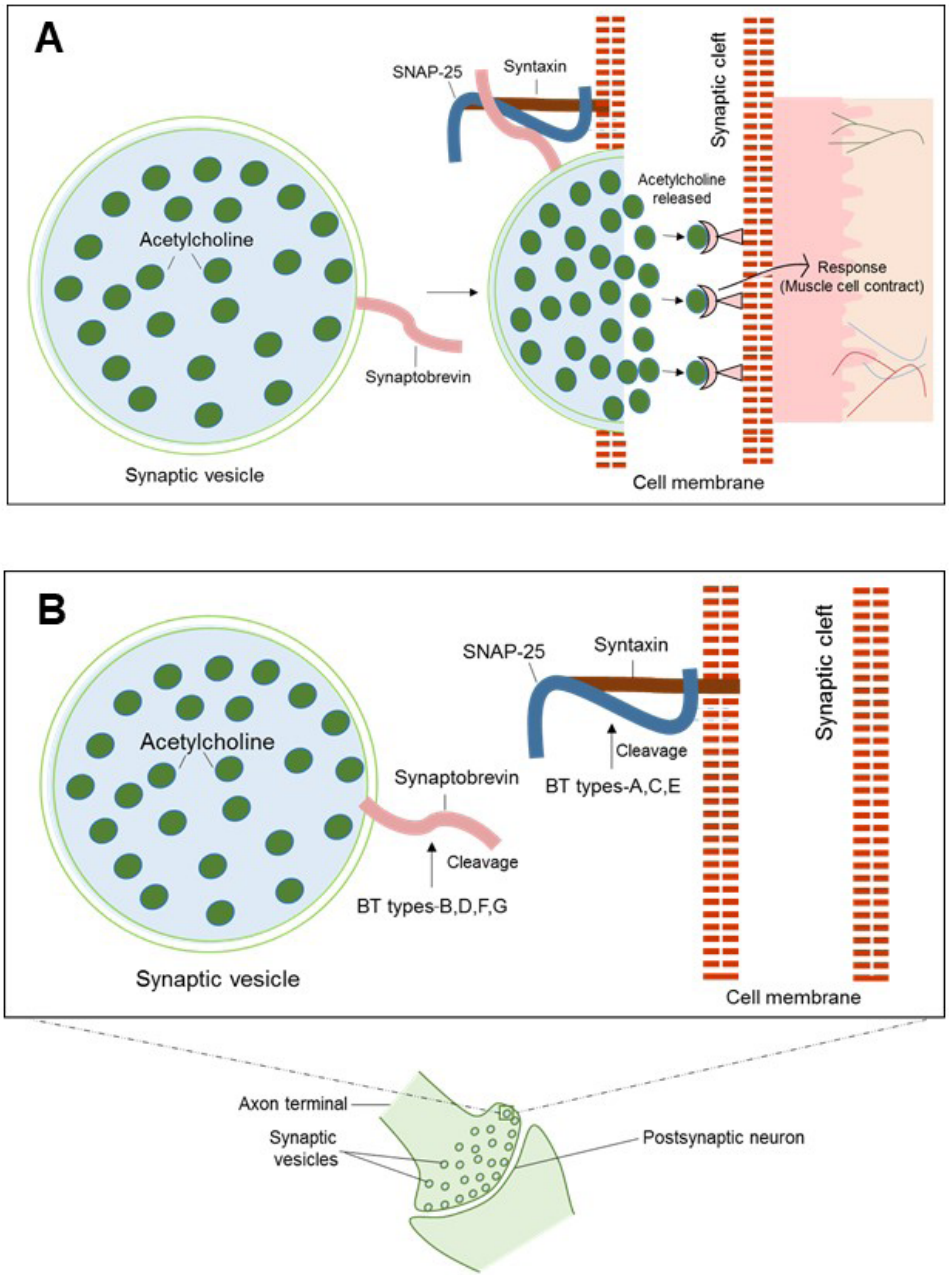

Fig. 1. Mechanism of acetylcholine release at the junction of neurons and muscles. (A) Normal condition. (B) Action of botulinum toxin to prevent the release of acetylcholine into the synaptic cleft.

vents the release of acetylcholine into the synaptic cleft by blocking membrane fusion. Hence, muscle cells stop responding. Therefore, an individual exposed to botulinum toxin could experience muscle paralysis (Fig. 1B; Dressler *et al*., 2005). Botulinum toxins can cause a rare but life-threatening condition called botulism, characterized by weakness, blurred vision, speech impairment, muscle cramps, vomiting, diarrhea, and fever. The estimated human lethal dose of botulinum toxins is 1.3-2.1 ng/kg when administered by the intravenous or intramuscular route and 10-13 ng/kg when administered by the inhalation route (Alshadwi *et al*., 2015; Lentz and Weingrow, 2018).

MEDICAL USES OF BOTULINUM TOXIN

Although botulinum toxins are considered to have high toxicity; however, in low doses, they have been extensively used to treat various clinical conditions. Among the seven different types of botulinum toxins, types A and B are most commonly used for medical purposes. Some medical uses are as follows.

Muscle spasticity

Botulinum toxin is used to treat several overactive muscle disorders, including post-stroke spasticity, spinal cord injury associated spasticity, head and neck spasms, and clenching

of muscles of the esophagus, jaw, urinary bladder, and anus (Snow *et al*., 1990).

Excessive sweating

Acetylcholine facilitates sympathetic neurotransmission in the sweat glands. Bushara *et al*. (1996) and Heckmann *et al*. (2001) reported that injections of botulinum toxin A could inhibit excessive sweating by preventing the release of acetylcholine (Heckmann *et al*., 2001). In fact, the United States Food and Drug Administration (FDA) has approved botulinum toxin type A for use as a topical agent (Collins and Nasir, 2010).

Migraine

In patients with migraine, injection of low-dose purified botulinum toxin around the pain fibers prevented the release of chemicals involved in pain transmission and reduced the incidence of migraine (Silberstein *et al*., 2000). In 2010, FDA approved botulinum toxin injection for treating chronic migraine headaches (Escher *et al*., 2017).

Overactive bladder

Patients with an overactive bladder could be treated with a botulinum toxin injection into the walls of the urinary bladder, which reduces the urge for frequent urination (Duthie *et al*., 2011). Botulinum toxin prevented the vascular release of acetylcholine, reducing urinary bladder contraction and benefiting patients with a refractory overactive bladder. Thus, FDA has approved the use of botulinum toxin injection for an overactive bladder (Cox and Cameron, 2014).

Facial wrinkles

Botulinum toxin is considered safe for reducing facial wrinkles (Benedetto, 1999; Carruthers and Carruthers, 2002). It has been efficacious in relaxing wrinkled muscles, resulting in a smooth overlay skin; moreover, superior results were observed after few repeated injections (Benedetto, 1999; Carruthers and Carruthers, 2002). Notably, botulinum toxin selectively binds to the peripheral cholinergic motor neuron endplates to prevent the release of acetylcholine. Consequently, it paralyzes the involved muscles for a short period of up to 3 months (Small, 2014). However, the restoration of muscle functions can be observed shortly after the gradual formation of new motor endplates (Dressler *et al*., 2005).

IN VIVO TESTING OF BOTULINUM TOXINS FOR MEDICAL USE

Mouse lethality bioassay (MLB)

For botulinum toxin products used for medical purposes, animal testing has been exclusively employed for assessing efficacy and safety. The *in vivo* MLB is a standard test to evaluate the potency of botulinum toxin (Dressler *et al*., 2000; Lindström and Korkeala, 2006). In this assay, the biological activity of a sample is compared with that of standard samples. For decades, the LD_{50} assay has been the only method to determine the safety and potency of each batch of botulinum toxin manufactured for medical and cosmetic uses. Different doses of botulinum toxin are injected intraperitoneally into mice to assess mortality. Based on animal deaths observed in each group, the potency of botulinum toxin is calculated (Dressler *et al*., 2000; Lindström and Korkeala, 2006). MLB is very sensitive and has been reported a LD_{50} of 5-10 pg/mL for botulinum toxin (Dunning *et al*., 2014).

Limitations of MLB

The test endpoint of botulinum toxin testing is the painful death of animals following respiratory failure. Therefore, using a large number of animals for the efficacy/toxicity testing of botulinum toxins would be in disagreement with the 3R concepts (Reduction, Replacement, Refinement) adopted by the European Union and the Organization for Economic Cooperation and Development, which suggests the development of alternative test methods rather than using animals for such studies (Törnqvist *et al*., 2014). Scientists face an ethical dilemma to test botulinum toxins in animals as it overrules the scope of the aforementioned 3R concept by inducing distress and pain in animals, as well as inhumane practice that leads to death (Kang *et al*., 2018). Additionally, animal testing is a laborious and an expensive procedure requiring a sophisticated animal facility and a skilled and dedicated workforce. It has been estimated that for testing a single sample of botulinum toxin by MLB, 6-16 mice are required, with approximately 10 min for sample preparation and 2 days for toxicity manifestation in animals. However, this method has not been effective in detecting botulinum spores. Therefore, the occurrence of false positive results for *C. botulinum* spores would be high, creating a high chance of misreading results and false data interpretation.

In these regard, in recent years, there has been substantial progress regarding botulinum toxin testing in animals in Europe. However, these developments are still dependent on animal tests, inevitably causing severe pain and requiring a large number of animals. In addition, the paradigm for research has been evolving; human benefits do not justify harming animals anymore. Therefore, researchers have attempted to develop alternative testing methods for the safe use of botulinum toxin in humans (Taylor *et al*., 2019). Some of the currently available alternative methods were compiled in this review.

ALTERNATIVE METHODS DEVELOPED FOR BOTULINUM TOXIN TESTING

SNAP-25 assay

The sensitivity (<10 pg/mL) of this method is similar to that of the mouse bioassay. This assay system is faster, more automated, and can be adapted to several laboratory settings (Rasooly and Do, 2008; Yadirgi *et al*., 2017). During poisoning, the light chain of botulinum toxins selectively cleaves the intracellular synaptosome-associated protein of molecular mass 25-kDa (SNAP-25) (Keller and Neale, 2001). Scientists utilized this distinct mechanism and developed an *in vitro* method to measure the cleavage of SNAP-25 by employing fluorescence detection methods (Rasooly and Do, 2008; Yadirgi *et al*., 2017). The assay is performed in two simple steps. First, the toxin is immuno-separated and concentrated using immuno-magnetic beads with monoclonal antibodies directed against the 100-kDa heavy chain subunit. Second, the SNAP-25 peptide is cleaved by the toxin, labeled with fluorescent dyes, and detected by fluorescence resonance energy transfer-based techniques. The schemes for the detection of botulinum toxins A and E are illustrated in Fig. 2. This technique is very effective as an alternative for animal use in botu-

linum toxin testing (Yadirgi *et al*., 2017). When SNAP-25 is cloned to express large quantities in the pure form, animal use could be completely banned. In this assay, owing to the use of immune-magnetic beads, the botulinum toxin of interest could be concentrated in the given sample, demonstrating a sensitivity as high as that of MLB. However, this method does not involve all the critical steps of botulinum toxin poisoning, such as binding, internalization, and intracellular activity, and might lead to false results. Recently, with the development of a comprehensive panel of highly specific monoclonal neo-epitope antibodies, researchers could simultaneously detect at least two botulinum toxin serotypes (von Berg *et al*., 2019). The advances in the field of SNAP-25 assay could be considered a major step toward the replacement of MLB.

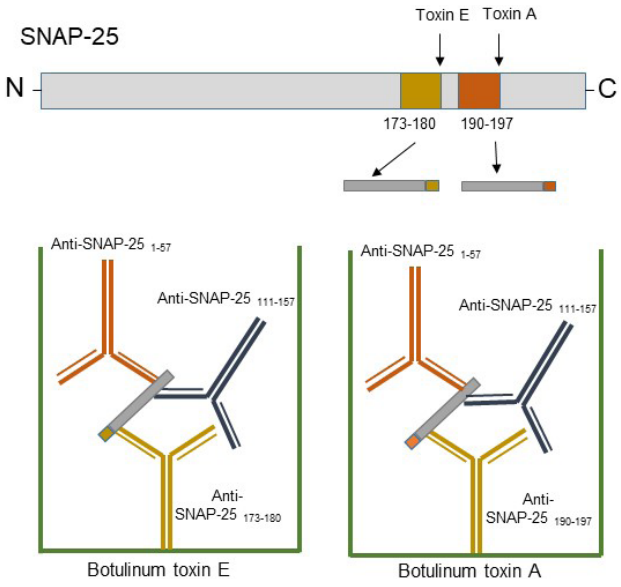

Fig. 2. Schematic overview of immune-detection of toxin-cleaved SNAP-25. Toxin A cleaves SNAP-25 in neurons between the 197th and 198th amino acids, and toxin E cleaves between the 180th and 181st amino acids. The cleaved fragments bind to their specific antibodies and are caught by neo-epitope antibodies produced against the peptides corresponding to SNAP-$25_{190-197}$ and SNAP-$25_{173-180}$. The antibodies only detect the toxin-cleaved SNAP-25 fragment and would not bind to intact SNAP-25. The captured cleavage product is then detected using two polyclonal detection antibodies that bind to two distinct sites, SNAP-25_{1-57} and SNAP-$25_{111-157}$.

Ex vivo assays

Mouse phrenic nerve hemidiaphragm (MPN) test: The MPN test is an *ex vivo* study employing isolation of the hemidiaphragm muscle with the attached phrenic nerve from euthanized mice. This test was first described by Bülbring in 1946 using the rat phrenic nerve and was later adopted and modified using the mice phrenic nerve (Bülbring, 1946; Bigalke and Rummel, 2015). Although a dramatic increase in sensitivity was observed from rats to mice, the observed paralytic half time was similar (Bigalke and Rummel, 2015). This assay closely imitates MLB by mimicking *in vivo* respiratory paralysis. The phrenic nerve originates in the neck (C3-C5) and passes down between the lung and heart to reach the diaphragm. The use of both halves of the diaphragm could reduce the animal use by half; however, as the right phrenic nerve is present behind vital organs and is closely attached to main blood vessels, this method uses only the left phrenic nerve hemidiaphragm for successful dissection (Bigalke and Rummel, 2015). In this assay, the excised phrenic nerve is placed in an organ bath maintained with optimized pH, O_2, and CO_2 levels and is continuously electro-stimulated at a frequency of 1 Hz with two electrodes. Then, the isometric contraction amplitude is measured to analyze the data obtained. Next, the incubation solution is replaced with the botulinum toxin-containing solution and the reduction in contraction amplitude is measured. The time required for the reduction of 50% amplitude is determined as the assay endpoint (Bigalke and Rummel, 2015). In this method, botulinum toxins (A, B and E) demonstrated a dose-dependent decrease in the contraction amplitude of the stimulated muscle. An excellent correlation of 0.96-0.99 was achieved between MPN and MLB for all botulinum toxins tested (Table 1; Rasetti-Escargueil *et al*., 2011).

Table 1. Summary of the methods for detecting botulinum toxins

	MLB	SNAP-25 assay	MPN	NFPA	Immuno-assays	Catalytic activity assays	Cell-based assay	Nucleic acid-based assay
Sensitivity (pg/mL)	<10[a]	(0.3-80)[b]	(30-50)[c]	<10[a,d]	(0.2-2.2)[e,f]	(0.1-1,000)[g]	~3[h]	(1-5)[i]
Duration (including sample preparation time, day)	>5[j]	<1[j]	<1[j]	>2[j]	<1[j]	<1[j]	Variable[j]	(1-2)[j]
Correlation with MLB	-	0.95[k]	(0.96-0.99)[l]	0.98[m]	0.94[n]	(0.85-0.97)[o]	N/A	N/A
Serotypes detected	(A, B, C, D, E, F, G)[p]	(A, B, C, D, E, F, G)[b]	(A, B, E)[c]	(A, B)[q]	(A, B, E, F)[r]	(A, B, E, F)[s,t]	(A, B, E)[u]	(A, B, E, F)[v]
Experimental design	*in vivo*	*in vitro*	*ex vivo*	*ex vivo*	*in vitro*	*in vitro*	*in vitro*	*in vitro*

MLB, mouse lethality bioassay; MPN, mouse phrenic nerve hemidiaphragm test; NFPA, non-lethal mouse flaccid paralysis assay; N/A, not available.

References: [a]Wictome *et al*. (1999); [b]von Berg *et al*. (2019); [c]Bigalke and Rummel (2015); [d]Wilder-Kofie *et al*. (2011); [e]Cheng and Stanker (2013); [f]Sharma *et al*. (2006); [g]Kalb *et al*. (2015); [h]Rust *et al*. (2017); [i]Čapek and Dickerson (2010); [j]Stephens (2005); [k]Ekong *et al*. (1997); [l]Rasetti-Escargueil *et al*. (2011); [m]Sesardic *et al*. (1996); [n]Zechmeister *et al*. (2002); [o]Björnstad *et al*. (2014); [p]Dunning *et al*. (2014); [q]Sesardic and Das (2007); [r]Ferreira (2001); [s]Rosen *et al*. (2017); [t]Boyer *et al*. (2005); [u]McNutt *et al*. (2013); [v]Cheng *et al* (2016).

Although animals are inevitably sacrificed to prepare the phrenic nerve, this is an improved test owing to the reduced animal use. MPN can determine the presence of botulinum toxins, along with their efficacy, potency, and concentration in the given sample. Therefore, this method could be considered more precise than MLB. However, it requires experienced and skilled personnel and only a limited number of samples can be analyzed in a single assay. Moreover, as the test only identifies active botulinum toxins, if the samples contain inactivated or denatured toxins and other muscle-paralyzing agents, the test may produce false results (Bigalke and Rummel, 2015).

Non-lethal mouse flaccid paralysis assay (NFPA): NFPA is an *ex vivo* local paralysis assay that is considered less severe, more economical, more sensitive, and a refinement of the mouse LD_{50} assay. This assay uses mouse paralysis as the endpoint to determine the potency of botulinum toxin type A (Sesardic and Das, 2007). The extent of paralysis reflects the potency of the toxin. This method evaluates exposure to botulinum toxin type A by employing the stimulated rodent diaphragm or rat intercostal muscle. This method has several advantages over the conventional method, MLB. Here, the endpoint is more humane compared with that in MLB, with only a 4% reduction in animal body weight observed during the test (Sesardic and Das, 2007). Furthermore, in this method, the endpoint is evaluated locally, thus avoiding systemic toxicity, including death in mice. Moreover, the endpoint of paralysis can be observed within 24-48 h, which is considerably shorter than 72-96 h required to observe the endpoint in acute toxicity (LD_{50}) testing. This method has been validated at the National Institute for Biological Standards and Control (UK) and has been accepted as an alternative method to test the potency of botulinum toxin in the European Pharmacopoeia (Sesardic and Das, 2007).

Furthermore, to perform this method, no specialized equipment is required, and only 20% of the animals used for MLB are used for this assay (Sesardic and Das, 2007). A correlation of 98% was achieved when compared with MLB. Additionally, the mean difference between the estimated potency in the two assays was not statistically significant (Sesardic *et al*., 1996). A linear relationship was achieved between the mean scores of response vs toxin doses, which proved that the developed method was sensitive and highly efficient for the determination of botulinum toxins. With the confidence interval of 95%, the geometric coefficient of variation within assays was achieved as 16%. In this method, the accuracy and precision obtained using sub-lethal doses of botulinum toxin were comparable to the MLB, confirming that the FDA and other regulatory agencies can accept the assay method as a replacement for MLB.

Immunoassays

Immunoassays offer the simple, quick, sensitive, and reproducible detection of botulinum toxins, providing both qualitative and quantitative evidence (Lindström and Korkeala, 2006; Thirunavukkarasu *et al*., 2018). These measure the interaction between the protein antigen from pathogenic organisms and the antibody (Lindström and Korkeala, 2006; Sharma *et al*., 2006; Rasooly and Do, 2008). Classical enzyme-linked immunosorbent assay (ELISA) has been employed for the detection of botulinum toxins. However, the sensitivity of detection for botulinum toxins obtained by employing standard ELISA was moderately less than that obtained using mouse bioassays (Lindström and Korkeala, 2006; Sharma *et al*., 2006; Rasooly and Do, 2008). Nevertheless, signal amplification methods, such as the chromogenic diaphorase system, have been utilized to increase the sensitivity to that of MLB (Lindström and Korkeala, 2006; Sharma *et al*., 2006; Rasooly and Do, 2008). Usually, monoclonal or polyclonal antibodies are used for the detection of various serotypes of botulinum toxins (Cai *et al*., 2007; Čapek and Dickerson, 2010). In contrast to MLB, immunoassays have excellent dynamic ranges of quantification based on sample dilution. Furthermore, immunoassays are cost-effective, requiring fewer instruments that do not need skilled personnel. A major limitation of this method is the use of high-quality antibodies, which are costly and difficult to produce. Moreover, chances for false positive results are relatively high owing to the heat-inactivation of toxins (Lindström and Korkeala, 2006). Additionally, the sensitivity of assay varies between samples and botulinum toxin serotypes. The detection limit for botulinum toxin type A, B, E, and F by ELISA techniques was calculated as 60, 176, 163, and 117 pg/mL, respectively (Lindström and Korkeala, 2006; Sharma *et al*., 2006; Rasooly and Do, 2008). The tests readily detected 2 ng/mL of serotypes A, B, E, and F in a variety of tested foods (Cheng *et al*., 2012). In addition, with the development of high-affinity antibodies, ELISA based systems could detect botulinum toxins serotypes A, B, C, D, E, and F with concentrations ranging from 0.2 to 2.2 pg/mL (Zhang *et al*., 2012). FDA has accepted ELISA for the detection of botulinum toxins A, B, E, and F (Food and Drug Administration, 2017). However, positive samples determined using ELISA need to confirmed by MLB.

Another immunoassay approach is the electro-chemiluminescence (ECL) method (Cheng and Stanker, 2013). The ECL approach uses a format similar to ELISA. The output signal is produced by enzymatic hydrolysis of certain substrates in ELISA; however, ECL uses a luminescent signal generated by electron cycling of the ruthenium label. In this method, electrochemically generated intermediates undergo a highly exergonic reaction to yield an electronically excited state discharging light upon relaxation to a lower-level state (Forster *et al*., 2009; Valenti *et al*., 2016). The ECL microplate consists of a carbon electrode surface that uses ruthenium labeled antitoxins. When these antibodies detect botulinum toxins, luminescence occurs (Cheng and Stanker, 2013). Briefly, the ECL standard samples in a 96-well plate are treated with anti-botulinum toxin antibodies, and the amount of toxin present in each sample is determined by comparing the unknown signal to the standard curve signal. In this method, the limit of detection was 3 pg/mL for botulinum toxin-serotype A and 13 pg/mL for botulinum toxin-serotype B (Cheng and Stanker, 2013). However, such methods only provide information on the amount of protein and do not reflect the biological potency of toxins.

Assays for the catalytic activity of botulinum toxin

Botulinum toxins can be detected and identified by determining the catalytic activity of their endopeptidase domain (Parks *et al*., 2011). Theoretically, every botulinum toxin has a unique substrate cleavage site(s). Hence, an *in vitro* assay that could identify the specific target substrate and endopeptidase activity could be utilized to determine the specific botulinum toxin (Björnstad *et al*., 2014; Rosen *et al*., 2015). Recently, the Endopep-MS assay using a combination of botulinum toxin endopeptidase enzyme activity with mass spectrometry was developed to determine the specific location of the cleaved

substrate (Björnstad *et al*., 2014; Rosen *et al*., 2015). This test effectively determines botulinum toxin levels in clinical samples, food samples, and cultures. In addition, this assay system is rapid, reliable, and robust to detect and differentiate various serotypes of botulinum toxins (Björnstad *et al*., 2014; Rosen *et al*., 2015). Kalb *et al*. (2015) recently developed an assay system that incorporates serotype-specific, high-affinity monoclonal antibodies for binding to the heavy chains of different botulinum toxins. This enables the Endopep-MS assay to attain higher sensitivity that is comparable with or more sensitive than the conventional method MLB (100 fg/mL-1 ng/mL) (Kalb *et al*., 2015).

Recently, researchers assessed the enzymatic activity of botulinum toxin by using immunoassay techniques including ELISA, which was capable of detecting three botulinum toxin serotypes, A, B, and E (Rhéaume *et al*., 2015; Simon *et al*., 2015). The Endopep-ELISA uses monoclonal antibodies that do not bind with substrate molecules in the uncleaved state, binding specifically to the new binding site of epitopes, generated following the cleavage of target substrates (Wictome *et al*., 1999; Nuss *et al*., 2010). The sensitivity obtained with this method was comparable to or even exceeded MLB (Rhéaume *et al*., 2015; Simon *et al*., 2015). This method can evaluate botulinum toxin levels in clinical samples, food samples, and cultures; however, this method identifies only a few serotypes (Kalb *et al*., 2015).

Cell-based assay

Cell-based assays are viable *in vitro* options that could detect fully functional botulinum toxins in a single assay. Over the past 5 years, assays for several botulinum toxins based on cell levels have been established, with the potency of botulinum toxins quantitatively evaluated with a similar or higher tolerance than the mouse bioassay (Pellett, 2013). Stem cell- and neurogenic cell line-based assays have been used for the identification of biological activities of the botulinum toxins (Maslanka *et al*., 2011; McNutt *et al*., 2013; Thirunavukkarasu *et al*., 2018). Stem cell- or neurogenic cell line-based assays offer comparable sensitivity to MLB for the detection of botulinum toxin. However, the time period required for performing the assay was similar to the MLB. In this assay system, cells are incubated with botulinum toxins for a defined period (24-72 h), followed by the removal of toxins, and the determination of toxin activity in cells (Pellett, 2013). To quantitate the toxin activity in cells, the cleavage of SNARE proteins was determined by either ELISA or Western blotting in cell lysates (Pellett, 2013). Alternatively, the toxin activity could be determined in live cells by immune-fluorescence methods by using cleavage-specific antibodies (Kiris *et al*., 2011). Another significant, but less precise endpoint, is the determination of the release of neurotransmitters, that can be assessed in primary neuronal cell cultures and neurons originating from stem cells, as well as in certain continuous cell lines (Bigalke and Rummel, 2015). For these assays, well-differentiated human or mouse neural cells, or embryogenic or induced pluripotent stem cells are required. The cell-based botulinum toxin test was validated and approved by the US FDA, Health Canada, and the European Union for testing botulinum toxin-based products (Fernandez-Salas *et al*., 2012). The main advantage of the cell-based assay system is reduced number of animals compared with MLB (Thirunavukkarasu *et al*., 2018). However, the major limitations of this method were sample-to-sample variation in results, limited number of samples in a single assay, and the requirement of skilled workforce with appropriate facilities for the cell studies (Thirunavukkarasu *et al*., 2018).

Table 2. Primers used for detecting specific botulinum toxin genes

Types	Genes	Primer sequences (5'-3')
Type A	BT(A)	
	Forward	AGCTACGGAGGCAGCTATGTT
	Reverse	CGTATTTCCAAAGCTGAAAAGG
Type B	BT(B)	
	Forward	CAGGAGAAGTGGAGCGAAAA
	Reverse	CTTGCGCCTTTGTTTTCTTG
Type C	BT(C)	
	Forward	CCAAGATTTTCATCCGCCTA
	Reverse	GCTATTGATCCAAAACGGTGA
Type D	BT(D)	
	Forward	CGGCTTCATTAGAGAACGGA
	Reverse	TAACTCCCCTAGCCCCGTAT
Type E	BT(E)	
	Forward	CCAAGATTTTCATCCGCCTA
	Reverse	GCTATTGATCCAAAACGGTGA
Type F	BT(F)	
	Forward	CGGCTTCATTAGAGAACGGA
	Reverse	TAACTCCCCTAGCCCCGTAT

BT, Botulinum toxin.

Nucleic acid-based methods

Several nucleic acid-based methods have been used to identify the presence of botulinum neurotoxin in clinical and environmental samples, food, and pharmaceutical products (Thirunavukkarasu *et al*., 2018). Using polymerase chain reaction (PCR), specific genes that encode bacterial toxins could be amplified, providing insights on the toxin-producing ability of the sample organism (Szabo *et al*., 1993; Lindström and Korkeala, 2006; Peck, 2006; Fach *et al*., 2009). Therefore, the botulinum toxin gene from different strains of *C. botulinum* can be amplified, and the type of toxicity imparted by each strain of bacteria can be estimated (Raphael, 2012; Smith *et al*., 2015). A series of PCR primers were designed, and genes that compose the toxin capable of producing toxicities were determined, as shown in Table 2 (Lindström *et al*., 2001; de Medici *et al*., 2009). However, this nucleic acid-based assay would only be effective in the presence of toxin-producing bacteria in tested samples. This method demonstrates a similar sensitivity to MLB. However, this method requires skilled workforce for using complex instruments and requires substantial time for analysis compared with other *in vitro* methods. The US FDA has accepted the PCR method for the detection of botulinum toxins A, B, E, and F (Food and Drug Administration, 2017). However, positive test samples determined by PCR should be confirmed by *in vivo* MLB.

FUTURE PERSPECTIVES

Millions of experimental animals are sacrificed every year to perform non-clinical tests. *In vivo* animal tests are deemed humane for determining the efficacy and toxicity of test substanc-

es. However, inhumane treatments that cause pain, distress, and death in animals are inevitable in animal experimentation, particularly in eye irritation and skin sensitization tests. As the European Union and the Organization for Economic Co-operation and Development have adopted the 3R concept in developing alternative tests, numerous test methods have been developed and implemented as alternatives to animal experiments. However, the development and implementation of such alternatives are still required in numerous fields where a large number of test animals are employed, particularly in testing the efficacy and toxicity of botulinum toxins, as animal death with pain and stress is the only endpoint for the evaluation of toxins. In the past decade, there have been significant strides in developing alternative techniques for the rapid and robust detection of botulinum toxins. However, more effective and less stressful methods are imperative for detecting minimal concentrations of toxins in test samples. In addition, such methods should be comparable with *in vivo* tests to evaluate the potency of toxins. Likewise, in the gold standard MLB, only a limited number of samples can be analyzed at a given time. The ability to detect multiple serotypes from complex sample matrices simultaneously would be a key requirement in the diagnostic and food testing sectors while maintaining the need for rapid and low-cost detection. Therefore, newly developed assay methods must be capable of determining toxin levels in a large number of samples both qualitatively and quantitatively and must be cost- and time-effective, sensitive, and accurate with regard to the quantitative potency of samples. In this review, we briefly listed the methods for the identification of botulinum toxins and described the most successful developments in this field. All test methods described in this review have the common goal to address the 3R concept and replace the *in vivo* MLB. Among the several, proposed alternative methods for the detection of botulinum toxins, some have better sensitivity and response time than MLB, which could be a clear indication for developing alternative tests (Table 1). Moreover, we could also possibly replace MLB with one or a combination of alternative tests.

Furthermore, the assay method should be sensitive and proficient. The SNAP-25 assay is faster, automated, and could be adapted to many laboratory settings. However, antibodies in their purest forms are essential. *Ex vivo* assays, such as MPN test and NFPA, were successful in reducing the number of animals required for testing botulinum toxins; however, animals are still required in these tests. Conversely, immunoassays have excellent detection capability for all toxin serotypes; however, the potential for false positive results is relatively high owing to the possible presence of heat-inactivated toxins. Similarly, to determine the catalytic activity of botulinum toxins, assays such as the Endopep-MS assay and Endopep-ELISA were extremely promising with high sensitivity. However, only limited serotypes could be detected with these methods. Cell- and nucleic acid-based assays are the viable *in vitro* options that could detect fully functional botulinum toxins in a single assay. These methods demonstrate the advantages of identifying the biological activities of botulinum toxins and offer comparable sensitivity to MLB. Collectively, there is a very high possibility that MLB could be replaced with equally sensitive and proficient *in vitro* methods. So far, US FDA has accepted amplified ELISA and PCR techniques for the detection of botulinum toxins. However, positive results from *in vitro* tests should be confirmed using *in vivo* MLB (Food and Drug Administration, 2017). Other methods are still being validated.

Most importantly, related law-making should be urgently expanded to the use of alternative tests that do not use laboratory animals in testing botulinum toxins. Since 2013, the European Union has enacted laws that prevent the sale of cosmetic products or individual components which have been tested using animal experiments. Currently, more than 37 countries, including Korea, have legally prohibited animal experiments for the development of cosmetics. As a result of these efforts, the use of alternative testing methods has greatly been increased in the field of cosmetic development. Therefore, in the quantitative and potency tests of botulinum toxins for quality control, a legislative effort for systematically using alternative testing methods should be provided to drastically reduce the use of experimental animals in related research fields and industries. Fortunately, as a part of the attempts to reduce the use of experimental animals, efforts to legislate a law to promote the use of alternative testing have been under preparation by the National Assembly, with the co-operation of the Ministry of Food and Drug Safety, a related academic society (i.e., the Korean Society for Alternatives to Animal Experiments), and animal protection groups in Korea. Recognizing that the sacrifice of experimental animals can no longer be justified for human welfare, we expect that all the related groups will eventually support this effort.

Finally, to encourage the widespread use of alternative tests for quantitative analysis and titer evaluation of botulinum toxins, a validation system for the testing methods should be well established to assess whether the developed assay is scientifically equivalent or more reliable than animal test results. This is because scientifically confirming the validity of an alternative test would be the only way to offset any public anxiety associated with the use of alternative tests rather than animals. The internationally recognized Korean Center for the Validation of Alternative Methods (KoCVAM) was established by the Korean government. It is necessary to urge the re-organization of the system to expand the functionality of this Center and to function stably and systematically. It is beyond question that the use of alternative testing methods will expand in the future. Hence, these efforts should be implemented as early as possible. It is a mission that can no longer be delayed.

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