Editorial

Botulinum Neurotoxin: Advances in Diagnostics and Healthcare Applications

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Botulinum neurotoxin (BoNT) is the most acutely toxic substance produced by a gram-positive, rod-shaped bacterium called *Clostridium botulinum* [1, 2]. It is a 150 kDa protein comprising of a 100 kDa heavy chain that is joined to a 50 kDa light chain by a disulfide bond. The light chain of BoNT is a protease, which attacks the SNARE protein at a neuromuscular junction, thereby preventing the vesicles (storing acetylcholine) from binding to the plasma membrane (where neurotransmitter is released). Therefore, it blocks the neuromuscular transmission by preventing the release of acetylcholine and causes paralysis of muscles in botulism. There are seven distinct BoNT types designated as A-G. BoNT A, B, E and F cause disease in humans, while C and D are responsible for diseases in cows, birds and other animals. BoNT was first reported by Justinus Kerner as a 'sausage poison' as it was often found on improperly handled, preserved or prepared meat products. In 1895, Emile van Ermengem first recognized and isolated it from home-cured ham that was implicated in botulism. *C. botulinum* is classified into four physiological groups (I-IV) based on its ability to digest complex proteins. Groups I (proteolytic) and II (non-proteoytic) are responsible for most human botulism outbreaks, while Group III cause diseases in animals. Group IV have not been associated with any human or animal disease.

BoNT is such a potential bioterror weapon that only a kilogram of it can wipe out the entire human population. The lethal dose of BoNT for humans is very low i.e. 1.3-2.1 ng/kg for intramuscular/intramuscular injection and 10-13 ng/kg for inhalation. It was extensively used by Iraq in the Persian Gulf war in 1991 on the warheads of missiles and bombs [3]. Several outbreaks of botulism have already been witnessed worldwide. But the improved hygiene and processing methods have led to a stable lower number of confirmed botulism cases in the EU, which typically range from 103 cases in 2010 to 132 in 2009 [4]. Moreover, there have been reports of contamination in widely distributed canned food products [5]. Recently, there was a worldwide recall of Fonterra dairy products based on a report about suspected *Clostridium* contamination, although it turned out to be a contaminant that is incapable of causing botulism. However, the incident demonstrates the devastating impact of contamination in commercially distributed products. Foodborne botulism is caused by improperly heated food prior to being canned, improperly cooked canned food or contamination of canned food. While infant botulism is caused by the presence of bacteria in the soil and dust. BoNT denatures at temperatures above 80ºC, but the anaerobic spores that produce BoNT are heat-tolerant and can survive boiling water for prolonged duration. The spores can even survive in very harsh environments and are very hard to kill. Therefore, many foods are canned with a pressurized boil in order to kill the spores.

Since 1960, BoNT had been employed for various therapeutic applications, such as treatment of crossed eyes (strabismus), uncontrollable blicking (blepharospasm), spasm of lower esophageal sphincter (achlasia) and excessive sweating (severe primary axillary hyperhidrosis). The injections of BoNT in very low doses, well-known commercially as Botox, have a forcasted global market of $ 2.9 billion by 2018, which will account for >50% of facial aesthetics' market [6]. It prevents the development of wrinkles for up to 4 months by paralyzing the facial muscles. It is the most extensive cosmetic operation being performed in US.

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Botox was approved by United States Food and Drug Administration (FDA) in 1989 for the treatment of strabismus, blepharospasm and hemifacial spasms in patients over 12 years old [7]. The use of BoNT type B (trade names 'Myobloc' and 'Neuroblock') was approved by FDA for the treatment of cervical dystonia in 2000. In 2002, the use of BoNT type A was approved by FDA for temporarily improving the appearance of moderate-to-severe frown lines between the eyebrows i.e. glabellar lines [8]. These improvements can last up to a few months. Similarly, FDA approved the use of onabotulinumtoxin A for the treatment of chronic migraines in 2010. BoNT has also been employed for the treatment and prevention of chronic musculoskeletal pain. However, some cases of Botox-related adverse reactions, such as respiratory failure and death, were reported in 2008. It was further observed that BoNT can cause swallowing difficulties, speech problems, respiratory disorders, muscle weakness, allergy and paralysis of wrong muscle group. Therefore, in 2009, FDA issued a mandatory warning to be stated on Botox's packing that the effects of BoNT may spread from the site of injection to other areas of the body, causing symptoms similar to those of botulism [9]. Presently, BoNT-based cosmetic and therapeutic products are manufactured by several companies, such as Allergan, Inc. (USA), Solstics Neurosciences, LLC (USA), Merz Pharma (Germany), Ipsen Ltd. (UK), Lanzhou Institute of Biological Products (China) and Medy-Tox Inc. (South Korea). There is no doubt that due to increase convenience and less pain, Botox injections have become so popular now-a-days, especially for reducing wrinkles on the face and make it look much younger. However, there is a need for repeated injections every few months to sustain these effects, which makes the botox therapy quite expensive.

There have been tremendous advances in the botulinum diagnostics during the last two decades [10, 11]. The diagnosis of botulism is done in laboratory by mouse bioassay, enzyme-linked immunosorbent assay (ELISA), lateral flow assay and mass spectrometry. Several other prospective assay formats have also been demonstrated, such as those based on electrochemiluminescence, immuno-polymerase chain reaction (PCR), biosensors, cellular-based assay and endopeptidase activity assay. However, the gold standard for the determination of BoNT type is the mouse bioassay, which is the only accepted method to confirm BoNTs. But it has several shortcomings as it is labor-intensive, takes about 4 days and requires animal facility in addition to highly skilled personnel. Therefore, it does not meet the criteria of an assay format that can be employed in case of real bioterror threats, which require rapid detection of BoNT at the point of need. Similarly, ELISA has been used for BoNT detection for many decades, but it has ~5-fold lesser sensitivity in

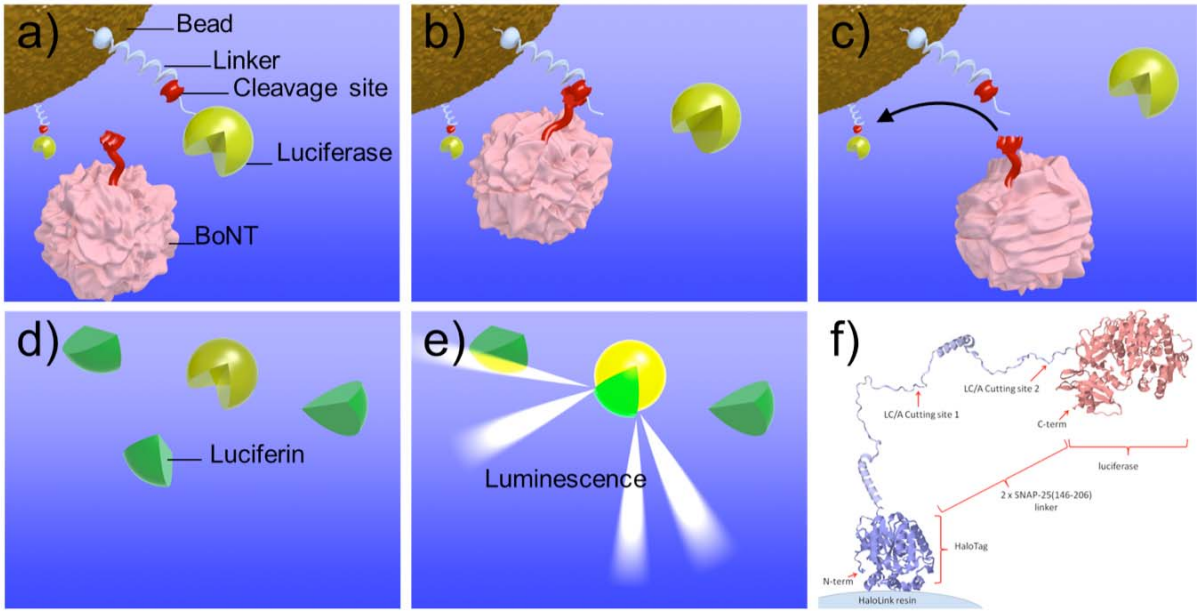

Figure 1: Schematic of the luciferase reporter assay for botulinum toxin [14, 15]. **a**) Firefly luciferase bound to beads by a polypeptide linker; **b**, **c**) BoNT hydrolyses the cleavage site of the linker, thereby releasing the luciferase; **d**) the beads are then removed followed by the addition of luciferin (in excess); **e**) luciferase reacts with luciferin substrate to produce the bioluminescence signal; and, **f**) 112 kDa fusion protein (HA-2SL) used. Reproduced with permission from Royal Society of Chemistry.

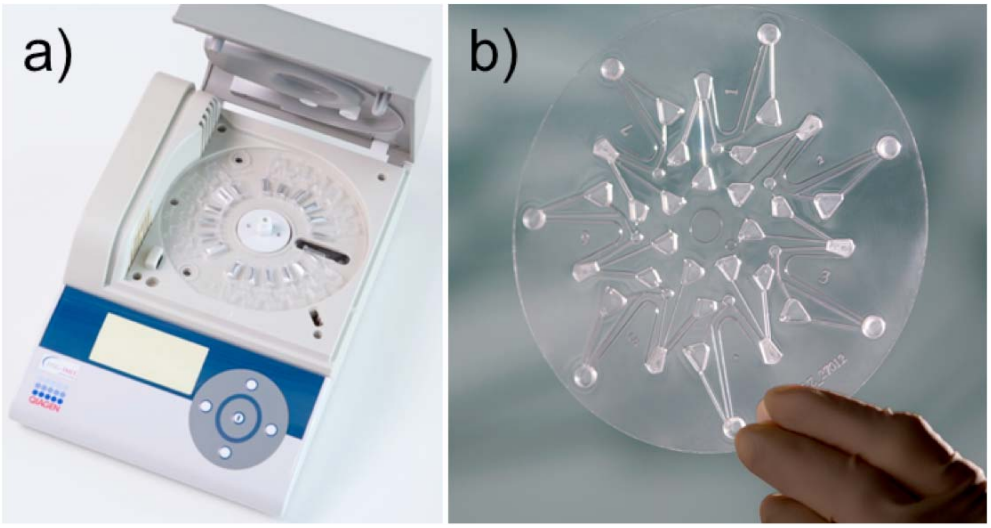

Figure 2: Developed luciferase reporter assay for the automatic detection of botulinum neurotoxin at point-of-need [15]. **a**) Portable centrifugal LabDisk player, **b**) disposable LabDisk.

comparison to mouse bioassay. The mass spectrometry-based BoNT assay is quite rapid (3-4 h) and can identify all BoNT types. Moreover, quantitative PCR can also be performed to differentiate the bacterial gene strains that produce BoNT types A, B, E, and F [12]. However, as BoNT is a highly toxic protein, it precludes other sensitive laboratory tests such as PCR and cell culture. The Naval Research Laboratory developed an array sensor for the rapid multiplex detection of BoNT type A and B in 20 min [13]. Recently, our group has developed two potential assay formats for BoNT detection [14, 15]. One format targets the highly sensitive detection of BoNT in central labs (Figure **1**), while the other format enables the fully-automated rapid detection of BoNT in just 20 min for point-of-need applications (Figure **2**). Both assays are based on the specific cleavage of a domain in a polypeptide linker by BoNT, where the cleaved polypeptides release luciferase, thereby producing a bioluminescence signal. The polypeptides were bound to microbeads, which enabled the automation of involved handling steps, such as mixing, washing and separation. However, despite these advances in the development of prospective assay formats for BoNT detection, there is still a critical need for improvements in analytical performance. The assay formats need to have higher sensitivity than the mouse bioassay and should be able detect all BoNT types.

The ongoing research efforts will pave way to the POC detection of BoNT, which will cater to the needs of emergency in case of bioterror attacks. On the other hand, taking into account the numerous advantages provided by Botox therapy, it will continue to be increasingly used in cosmetics and healthcare for diverse applications.

REFERENCES

[1] Shapiro RL, Hatheway C, Swerdlow DL. Botulism in the United States: A clinical and epidemiological review. Ann Intern Med 1998; 129: 221-8. http://dx.doi.org/10.7326/0003-4819-129-3-199808010-00011

[2] Gaya JG, Agni R, Miller JE. *Clostridium botulinum* and the clinical laboratorian. Arch Pathol Lab Med 2004; 128: 653-62.

[3] Bigalke H, Rummel A. Medical aspects of toxic weapons. Toxicology 2005; 214: 210-20. http://dx.doi.org/10.1016/j.tox.2005.06.015

[4] European Centre for Disease Prevention and Control. Annual Epidemiological Report 2012, ESDC 2013.

[5] Juliao PC, Maslanka S, Dykes J, *et al*. National outbreak of type A foodborne botulism associated with a widely distributed commercially canned hot dog chili sauce. Clin Infect Dis 2013; 56: 376-82. http://dx.doi.org/10.1093/cid/cis901

[6] http://www.companiesandmarkets.com/News/Healthcare-and-Medical/The-global-botox-market-forecast-to-reach-2-9-billion-by-2018/NI2991 (Accessed on 04.10.2013)

[7] http://www.fda.gov/downloads/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/DrugSafetyInformationforHeathcareProfessionals/UCM143989.pdf (Accessed on 04.10.2013)

[8] http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ucm080509.htm (Accessed on 02.10.2013)

[9] http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2009/ucm175013.htm (Accessed on 04.10.2013)

[10] Cai S, Singh BR, Sharma S. Botulism diagnostics: from clinical symptoms to *in vitro* assays. Critical Reviews in Microbiology 2007; 33: 109-25. http://dx.doi.org/10.1080/10408410701364562

[11] Lindstrom M, Korkeala H. Laboratory diagnostics of botulism. Clinical Microbiol Rev 2006; 19: 298-14. http://dx.doi.org/10.1128/CMR.19.2.298-314.2006

[12] Satterfield BA, Stewart AF, Lew CS, Pickett DO, Cohen MN, Moore EA, *et al*. A quadruplex real-time PCR assay for rapid detection and differnetiation of the *Clostridium botulinum* toxin genes A, B, E and F. J Med Microbiol 2010; 59: 55-64. http://dx.doi.org/10.1099/jmm.0.012567-0

[13] Ligler FS, Taitt CR, Shriver-Lake LC, Sapsford KE, Shubin Y, Golden JP. Array biosensor for detection of toxins. Anal Bioanal Chem 2003; 377: 469-77. http://dx.doi.org/10.1007/s00216-003-1992-0

[14] Stevens GB, Silver DA, Zgaga-Griesz A, *et al*. Bioluminescence assay for the highly sensitive detection of botulinum neurotoxin A activity. Analyst 2013; 138: 6154-62. http://dx.doi.org/10.1039/c3an00525a

[15] van Oordt T, Stevens G, Vashist SK, Zengerle R, von Stetten F. Rapid and highly sensitive luciferase reporter assay for the automated detection of botulinum toxin in the centrifugal microfluidic LabDisk platform. RSC Adv 2013. http://dx.doi.org/10.1039/c3ra44482a

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