

Fieldable Assay For Botulinum Neurotoxins

Ganapathy Rajaseger^{1*}, Padmanabhan Saravanan², Vernon J. Lee³, Vidhya Novem¹, Eric P. Yap¹, Shabbir Moochhala¹, Lee L. Hock¹, Ponnampalam Gopalakrishnakone^{1,2}

¹Defence Medical and Environmental Research Institute (DMERI), DSO National Laboratories (Kent Ridge), 27 Medical Drive, #09-01, Singapore 117510.

²Venom & Toxin research program, Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, and DMERI, DSO, Lower Kent Ridge Road, MD10, 4 Medical Drive, Singapore 117597.

³Headquarters Medical Corps, Singapore Armed Forces, 701 Transit Road, #03-01, Singapore 778910.

*Corresponding Author:

Ganapathy Rajaseger

DMERI, DSO National Laboratories

27 Medical Drive, #09-01

Singapore – 117510

Tel.: +65-64857211, Fax: +65-64857226, Email: grajaseg@dso.org.sg

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ABSTRACT

Background: The Botulinum neurotoxins (BoNTs) are a major threat in military settings. The mouse bioassay is the "gold standard" method, however, it has practical limitations. **Purpose:** To standardize a rapid, sensitive and fieldable optical immunoassay (OIA) for the detection of BoNTs A, B, E and F in food matrices. **Materials and methods:** The toxicity of BoNTs A, B, E and F was assessed by using a modified LD₅₀ assay. OIA was used to detect BoNTs in a variety of food matrices representing liquids, solids, and semisolid food. For specificity studies, the assay was used to test varying concentrations of epsilon toxin (ETX) from *Clostridium perfringens*, toxin A (TcdA) from *C. difficile* and staphylococcus enterotoxin B (SEB) from *S. aureus* in spiked assay buffer. To evaluate the OIA performance, five concentrations (serially five-fold diluted, viz. 20, 4, 0.8, 0.16 and 0.032 in ng/mL) of BoNT/A were made in orange juice and tested by both OIA and mouse bioassay. **Results:** The mouse intraperitoneal (i.p.) LD₅₀ values (in ng/kg) of BoNTs A, B, E and F were determined to be 1.6, 1.8, 1.3, and 2.4 respectively. OIA could readily detect 20ng/mL of BoNTs A, B, E and F in all the food samples tested. OIA demonstrate limits of detection lesser in range to the gold standard mouse bioassay. **Conclusion:** The OIA was found to

be rapid and sensitive in detecting BoNTs A, B, E and F from a variety of food matrices. Further enhancement of OIA sensitivity could be achieved using a toxin concentration protocol in food samples and use of an optical read-out device.

INTRODUCTION

Botulinum toxins produced by various *Clostridium* species are potent neurotoxins that block cholinergic neurotransmission [Ambache, 1951; Montecucco and Molgo, 2005]. The four botulinum neurotoxin (BoNT) serotypes BoNT/A, BoNT/B, BoNT/E, and BoNT/F account for almost all cases of human botulism [Simpson, 1996]. Symptoms of botulism are neurological and are characterized by acute, descending flaccid paralysis of muscles, beginning with the cranial nerves [Woodruff et al., 1992; Broussard, 2001]. Botulinum toxins are a public health concern because of they can occur naturally in food that has not been preserved or treated properly. Botulinum toxins have emerged as a major bioweapon threat to military operations, and against civilian populations, because they are extremely potent and lethal, but easily producible. As a biological weapon, BoNTs may be aerosolized as an inhalation threat or used as a deliberate food contaminant. Extrapolated data from primates to humans indicate that a lethal dose of crystalline type A toxin for a 70 kg human is approximately 90—150 nanogram (ng) by intravenous (i.v.) or intramuscular (i.m.), 70—90 ng by aerosolization and 70 microgram (μg) by oral route [Walt and Franz, 2000; Arnon et al., 2001; Scarlatos et al., 2005]. In addition to use as a poison, clinical preparations of BoNT/A are frequently used in the treatment of a number of neurological conditions [Naumann et al., 1998; Dolly and Aoki, 2006; Chaddock and Foster, 2007; Sheffield and Jankovic, 2007].

A rapid and sensitive assay to detect BoNTs in field settings is of paramount importance for determining deliberate intoxication with this toxin or other natural causes of food poisoning. Furthermore, serotype determination is necessary for appropriate therapeutic intervention. The gold standard diagnostic assay for clinical and food specimens is the mouse bioassay which is extremely sensitive, with a detection limit of 1 LD₅₀. It is generally estimated that 1 mouse LD₅₀/mL is equivalent to 10—20 picogram for the most active serotypes A, B, E and F [Wictome et al., 1999; Barr et al., 2005]. Nevertheless, it has limitations, such as the use of large number of animals, and the expensive, laborious and time consuming procedure (up to four days to complete). Several immuno-based and molecular diagnostic assays have emerged as sensitive alternatives to the mouse bioassay [Scarlatos et al., 2005; Lindstrom and Korkeala, 2006; Han et al., 2007]. Despite the fact that many of these technologies claim to be rapid, sensitive, and reliable, they have constraints of laboratory dependence and/or use of expensive instrumentation. Moreover, the sensitivities of the commercially available smart tickets were variable and were ineffective to discriminate between the four serotypes (A, B, E, and F) [Gessler et al., 2007].

To enable use in field conditions and to aid early detection of food intoxication for prompt intervention, a simple yet accurate test is needed. In this report, we describe the performance of our optical immunoassay (OIA) system in the detection of BoNTs spiked in assay buffer and few food matrices (liquid and semisolid) with lower limits of detection approaching that of the mouse bioassay.

MATERIALS AND METHODS

Toxin LD₅₀ determination

Purified BoNTs A, B, E, and F were purchased from Metabio Inc. (Madison, WI, USA). Aliquots of the toxins were stored at -80°C, and used immediately after thawing. Swiss albino mice weighing 20—30 g were procured from Laboratory Animals Centre, Sembawang, Singapore. The approximate LD₅₀ values for the four BoNTs were determined by using the method of Meier and Theakston [1986] with modifications as follows. Briefly, 30 mice were divided into groups of three mice each. Nine varying doses (range, 10—0.5 ng/kg) of each toxin diluted in 0.05M phosphate buffered saline (PBS, pH 6.4) containing 0.2% gelatin were prepared. Each group were injected intraperitoneally (i.p.) with 0.5mL of a dose and a control group was mock inoculated with only diluent (without toxin). The mice were observed for four days for signs of illness or death. In this study three mice were used per dilution to minimize the variability of survival time to yield a reasonable approximation of the LD₅₀ values. A linear regression line was plotted between dose (D, in ng/kg) and dose versus survival time (D/T, in ng/kg/h) for each toxin type. The y-intercept where the regression line intersects the ordinate is the smallest dose that kills 50% of the animals in an unlimited time and represents an approximate LD₅₀.

Toxin-specific antibodies and neutralization assay

Polyclonal antibodies to the four BoNTs A, B, E and F were purchased from Statens Serum Institut (Copenhagen, Denmark) and immunoglobulin-G (IgG) was purified using Econopac-Protein A columns (Bio-Rad, USA) and Amicon-ultra 100kDa MWCO desalting spin column (Millipore, USA). Toxin specific antibodies to the four BoNTs were prepared by affinity purification adopting the procedure previously described for venom toxin [Le et al., 2003]. In brief, toxin-specific antibodies were prepared by immuno-affinity chromatography of monovalent antitoxin antibodies with respective homologous toxin columns; and immuno-absorption of cross-toxin reacting antibody molecules with heterologous toxin columns. The resulting antibodies were toxin-specific as tested by ELISA.

Neutralization assays were performed as per the procedure outlined by Centers for Disease Control and Prevention [CDC, 1998]. Briefly, 1.25mL of each toxin type was added to a 5mL round bottom snap cap falcon tubes and mixed

with 0.25 mL of the four botulinum antitoxins (adjusted to neutralize 1×10^3 mouse LD_{50}/mL of toxins) by gentle swirling. After 30 min incubation at $37^\circ C$, 0.5 mL of the toxin-antitoxin mixture was injected i.p. to each mouse. Mice were observed for 72 hrs for symptoms of botulism and time of deaths was recorded.

Optical immunoassay (OIA)

The silicon assay system (SILAS-I™) was obtained from ThermoBioStar, Colorado, USA. Biotin N-hydroxysuccinimide ester and dimethyl sulfoxide (DMSO) were purchased from Sigma. Protein concentrations were determined by Lowry's method with protein determination kit from Bio-Rad Laboratories, CA, USA, using human immunoglobulin as standard for antibody estimation and bovine serum albumin as standard for other protein estimations. Toxin specific antibodies to BoNTs A, B, E and F were biotinylated and stored as aliquots at $-80^\circ C$ until use.

The assay is simple and based on the detection of variation in the reflected light from an optically-coated silicon surface. The immuno-binding events on the surface of the optical chip causes destructive interference of a particular wavelength of reflected light that changes in colour from gold to purple-blue depending on the thickness of the molecular film formed or the amount of toxin present in the analyte (figure 1A). The results are interpreted by simple visual read-out and compared to the signal strength reference card (figure 1B) to match the full reflection colour spectrum of this optical surface (according to the thickness of the layer formed on top of the optical surface) and scored from 1 to 27 that best corresponded to the signal strength of the result

The OIA test chip preparation was performed as follows. Toxin specific antibodies diluted ($2.5 \mu g/mL$) in phosphate buffered saline (PBS, pH 7.4) were manually spotted (a $30 \mu l$ spot volume occupies a diameter of ~ 5 mm as observed from the image analysis of the chip taken alongside a stainless steel ruler) in an orderly pattern on each chip of a 4-chip test strip mounted on high-density polypropylene support (Figure 2). Unoccupied sites were then blocked with 1% Blockaid™ diluted in PBS (pH 7.4) containing 0.5% Tween-20 (T-20). Internal positive control was center-spotted (~ 2 mm diam) on each chip, blocked as above, and after wash with wash buffer (PBS containing 0.05% T-20) the test strips were stored in sealed plastic box at $4^\circ C$ until use.

The sensitivity assay was performed for each toxin with a concentration gradient of 0— 1000 ng/mL to determine the detection limit in spiked assay buffer (PBS, pH 7.4). The assay was performed with $30 \mu l$ of analyte per spot for each dilution. Visual read-out of colour score was interpreted using the colour scale shown in figure 1B and scored semi-quantitatively as in table 1.

Cross-reactivity

Cross reactivity studies for serotype A toxin were performed in the presence of homologous antibodies to serotypes B, E and F following the OIA procedure described above using three dilutions (serial five-fold) of toxins (range, 100—4 ng/mL). The procedure was repeated for B, E and F toxins. Specificity of antibodies were assessed using three dilutions (serial five-fold, range, 100—4 ng/mL) of epsilon toxin (ETX) from *Clostridium perfringens*, toxin A (TcdA) from *C.difficile*, and staphylococcus enterotoxin B (SEB) from *Staphylococcus aureus*.

Sample preparation

Control samples of BoNTs A, B, E or F (range, 1.25— 20 ng/mL) were prepared in 0.5mL of assay buffer (pH 7.4). Five-milliliter liquid food samples of Orange juice and bottled water were spiked with 200 ng/mL of purified BoNT/A or BoNT/E and 400 ng/mL of BoNT/B or BoNT/F. After 30 min incubation at room temperature (26°C) with intermittent shaking by inversion, 0.5 mL of clarified supernatant was mixed with assay buffer (1:1) for liquid matrices. For semisolid and solid samples (porridge, minced pork, steamed fish), 10g of food samples were mixed with 1:4 volume (w/v) of assay buffer, homogenized using a handheld homogenizer (PowerGen 35, Fisher Scientific Pte Ltd, Singapore) and clarified at 4,000xg for 30 min at 4°C to remove solid particles. Subsequently, the supernatants were spiked with the various concentrations of BoNTs A, B, E and F and then analyzed by OIA for the detection limits.

Comparison of the OIA and mouse bioassay detection limits

Five concentrations (serial five-fold) of purified BoNT/A (20, 4, 0.8, 0.16, and 0.032 in ng/mL) were made in orange juice to evaluate the detection limits of both the OIA and mouse bioassay. For each toxin concentration tested, OIA was performed in triplicate as detailed previously. The reactions were scored for their colour reaction on a scale from 1 to 27 (figure 1B). The remaining test sample at each toxin concentration was then used for mouse bioassay, which were performed on the same day as the OIA. Two mice were used for each test sample. One was inoculated i.p. with 0.2 mL type A botulinum specific antitoxin. After 30min, both the antibody-protected and a non-protected mouse were inoculated i.p. with 0.5 mL (per 20g BW of mice, weight 22±2g) of the test sample. Mice were observed for signs of botulism for 4 days post inoculation. A positive test was recorded if the non-protected mouse died or showed signs of botulism and antibody protected mouse remained healthy. A negative test was recorded if both mice survived. The test was recorded inconclusive if both mice died. In between the positive and the first negative dilutions, additional two-fold dilutions were made.

STATISTICAL ANALYSIS

Statistical analyses were performed using Graphpad Prism version 4.01 for Windows (Graphpad software, Inc., CA, USA). The sample size of animals used for determining the LD₅₀ values of toxin types was small and an unpaired t-test was used to get the estimate of the difference in means between the test and control groups. One-way Analysis of Variance (ANOVA) was used to compare unspiked food with spiked food matrices that contained varying concentrations of toxins. The experiments were repeated three times, and results with P values <0.05 were considered statistically significant. The limit of detection (LOD) in different food matrices was defined as the smallest concentration of BoNTs giving a signal significantly different (P<0.05) from the negative control.

RESULTS

The present study was undertaken to develop a rapid and sensitive OIA for the detection of BoNTs A, B, E and F in different food matrices and to compare the detection limit of OIA with that of the gold standard mouse bioassay.

Toxin LD₅₀ determination:

We determined the approximate LD₅₀ of the four BoNTs A, B, E and F by a modified survival time method. This technique basically compares the injected doses of toxins with the observed survival times of experimental animals. The calculated dose versus survival time (D/T) for each animal (test group) is shown in table 2. The plot of calculated average dose versus survival time (D/T, in the abscissa) and dose (D, in the ordinate) for each toxin type as determined by mouse bioassay is shown in figure 3. The point of intersection on the ordinate along the regression line is the minimum i.p. LD₅₀ of toxin dose in an unlimited time. The regression equation for the dose versus survival time for each serotype was obtained by an average of three determinations (figure 3). There is no significant variation (ANOVA, P<0.05, P value = 0.9043; Bartlett's test for equal variances, P<0.05, P value = 0.9957) in the mean±SD of dose versus survival time (D/T) and LD₅₀ values among the four serotypes tested viz. BoNT/A (1.6±0.2), BoNT/B (1.8±0.2), BoNT/E (1.3±0.1) and BoNT/F (2.4±0.4). The present lethality assay was designed and established after carefully considering many factors that may influence the outcome of the experiment including (i) experimental animal (species, strain, age, weight, sex), (ii) methodology (application route, volume administered, ambient temperature, housing conditions) and (iii) toxins (batch to batch variations, storage).

Neutralization titre of purified toxin specific IgG:

Toxin specific antibodies were purified by affinity chromatography method. The antibodies tested against the four serotypes displayed specific toxin-neutralizing activity in 0.25 mL volume that neutralizes 50 × LD₅₀ of each toxin administered i.p. to mice.

Sensitivity of OIA:

The limit of detection (LOD) of OIA for the four BoNTs was performed using varying dilutions (0—1000 ng/mL) of each toxin in assay buffer. The LOD values obtained were 2.5 ng/mL for BoNT/A (~78.8 LD₅₀) and BoNT/E (~69 LD₅₀), 5 ng/mL for BoNT/B (~44.9 LD₅₀) and BoNT/F (~34.2 LD₅₀) (figure 4).

Cross-reactivity:

Cross-reactivity of the toxin specific antibodies was tested against three different concentrations (100, 20, and 4 ng/mL) of BoNTs (A, B, E and F), ETX, TcdA and SEB toxins. The study revealed each serotype specific antibody detected its corresponding toxin type with <0.9% cross reactivity between other toxin types (data not shown).

OIA of BoNTs in food matrices:

BoNTs A, B, E and F were detected using the standardized OIA in a variety of food matrices (liquid, solid and semisolid). The results obtained are shown in terms of average colour scores (triplicate readings) at tested concentrations (figure 5). The assay detected the presence of all the four toxins in all the matrices tested at 20 ng/mL. Difference in the mean OIA scores obtained for the various matrices tested with BoNTs A, B, E and F were analyzed by one-way analysis of variance (ANOVA) and Bartlett's test for equal variances. The analysis revealed no significant variation with the colour score obtained for BoNT/A (P<0.05, P value = 0.9126; Bartlett's test P value = 0.9879), BoNT/B ((P<0.05, P value = 0.9669; Bartlett's test P value = 0.9769), BoNT/E (P<0.05, P value = 0.8556; Bartlett's test P value = 0.9756), and BoNT/F (P<0.05, P value = 0.8648; Bartlett's test P value = 0.9525). OIA effectively measured 5 ng/mL for BoNT/A and BoNT/E and 10 ng/mL for BoNT/B and BoNT/F in bottled water. The detection limits observed in spiked orange juice (figure 5 and 6) and porridge were ~10 ng/mL for BoNT/A and BoNT/E and ~20 ng/mL for BoNT/B and BoNT/F. All the four toxins were detected at 20ng/mL in pork and fish.

We compared the OIA with the mouse bioassay in which assays were carried out with food extract (orange juice) was spiked with a range of BoNT/A concentrations (table 3). Using a sample volume of 0.03mL, the lowest concentration range of toxin detected by OIA was 10-20ng/mL compared to 0.032ng/mL by the mouse bioassay (using typical volume of 0.5mL). Data obtained in these experiments showed that mouse bioassay was approximately more than ten-fold more sensitive than the OIA.

DISCUSSION

BoNTs are the most poisonous substance known and is responsible for the severe food intoxication in humans termed botulism. Based on its extreme toxicity, they are considered as a potential bioterrorism agent. Thus, rapid, sensitive and fieldable detection methods are needed to prevent the impact of its occurrence. Mouse bioassay is still considered the gold standard method for

detection and serotyping of BoNTs [Kautter and Solomon, 1977; Schantz and Kautter, 1977]. Even though this method does not measure the natural route of intoxication, it is the widely accepted method to measure the biologically active toxins. It has been shown that the use of survival time (time-to-death) assay can provide precise estimates of the LD_{50s} of botulinum toxin activity with fewer numbers (~8 - 10) of experimental animals with the same range of statistical variability as the classical method which employ 30 or more animals [Boroff and Fleck, 1966; Meier and Theakston, 1986; Pearce et al., 1994]. Many factors can be associated with variation in LD₅₀ estimates [for details see Zbinden and Fluri-Reversi, 1981] by different laboratories, and that majority of it was attributed to the response of mice to toxin [Schantz and Kautter, 1977; Zbinden and Fluri-Reversi, 1981].

In the present study most of the factors that affect LD₅₀ determinations were kept under control and the estimates of LD₅₀ (ng/kg) was determined to be 1.6 for BoNT/A, 1.8 for BoNT/B, 1.3 for BoNT/E and 2.4 for BoNT/F using the survival time assay. However, it was suggested that LD₅₀ determinations by survival time assay do not provide adequate measure of the biological potency of clinical preparations of botulinum toxin [Pearce et al., 1994].

Previous documentations signify the exploitation of OIA platform in the detection of pathogens, brain trauma markers, and snake venom toxins [Ettinger et al., 1999; Dong et al., 2002; Aldous et al., 2005]. In this study, we report data on standardization of an OIA that is rapid, sensitive, fieldable and could be used to detect BoNT/A, BoNT/B, BoNT/E and BoNT/F in assay buffer and spiked food matrices. The detection limit of OIA was determined to be 2.5-5ng/mL in spiked assay buffer, followed by 5-10ng/mL in water and 10-20ng/mL in orange juice, porridge, pork and fish samples. Part of the study data on the detection limit of BoNTs by OIA was presented in the NBC symposium [Rajaseger et al., 2009].

A number of in vitro assays most of which are immunobased have been investigated as alternatives to the mouse bioassay [Doellgast et al., 1993; Ferreira et al., 2003; Gessler et al., 2005; Rivera et al., 2006; Sharma et al., 2006]. Despite the fact that these assays contemplate the antigenicity of the sample and not the biological activity, they could remain as an important diagnostic tool in situations where the activity does not need to be established. Nevertheless, there are very few assays that are fieldable, but, have presented with variable sensitivities. An effective comparison between the OIA and the gold standard mouse bioassay would require inoculation of large number of animals at different dosage levels. To minimize the use of animals and to provide an analysis of the detection limits, we restricted our comparison to one food matrix (orange juice) using known concentrations of BoNT/A. The sensitivity of OIA was approximately ten fold less compared to that of the mouse bioassay. However, we must emphasize that this comparison between the two assays was not done volume to volume, the OIA used a sample volume of 0.03 mL compared to the typical volume (0.5 mL) used for mouse bioassay. The lowest limit of BoNT/A

detection obtained in orange juice by OIA was ~10 ng/mL. In a study, larger sample volume used in ELISA has yielded with greater sensitivity by approximately four fold [Rocke et al., 1998].

The time needed for the assay remains a limiting factor in field analysis, we found OIA to be substantially rapid with a turn around time (TAT) of ~35min. Other operational benefits of the present fieldable assay system include less logistic load, and simultaneous semi-quantitation, visual read out, and non-powered. Also, this approach could be readily adapted to the detection of all BoNT serotypes in a single test by using a customized test strip of specific antibody coated OIA chips for the BoNT serotypes and use of pooled labeled antibodies as detector reagents. Further analysis on a variety of spiked food matrices, and clinical samples, and storage validation will be necessary to substantiate the versatility and endurance of the assay.

The present OIA system offers a valuable compromise of sensitivity and time in the detection of BoNTs from food matrices when compared to assays dependent on sophisticated equipments such as electrochemiluminescence [Rivera et al., 2006] (50-400pg/ml, 2h), endopeptidase coupled with mass spectrometry [Barr et al., 2005] (0.31-0.62mLD₅₀U/ml, 2h), time-resolved fluorescence [Peruski et al., 2002] (200 pg/mL, 2 h 30 min), array biosensor [Sapsford et al., 2005] (20 ng/ml, 30 min) and DIG-ELISA (2 ng/ml, 4 h 30min) [Sharma et al., 2006]. One of the pre-requisite for OIA is the availability of a white light source to observe and score the results. The diameter of the developed spot is larger enough to be scored with naked eye. Optionally a magnifier could be used under a white light source to record the results. In addition, differentiation of weak positives from negative samples should be done with greater caution. In general, individuals with color vision deficiencies (dichromacy) would not be able to read the results and refer the color score card for semi-quantitation.

In summary, we have developed a rapid and sensitive OIA for the detection of BoNTs A, B, E and F, with assay duration of ~35min. The detection limit achieved by OIA was lower when compared to that of the mouse bioassay. We are continuing our work to improve the sensitivity of the OIA using a toxin concentration procedure and to integrate an optical read-out device. This study outcome could parallel the diagnostic efficiency of mouse bioassay and would further surpass the limitation of users with colour vision deficiencies.

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TABLES

Table 1: Semi-quantitation of the four botulinum neurotoxins (BoNTs) A, B, E and F was performed. Purified toxins were double diluted (1000—0.195 ng/mL) in assay buffer to determine the limit of detection and subsequent scoring for semi-quantitation. The semi-quantitative levels were obtained from the standard curve in figure 4.

Color Score	Grade	Toxin concentrations in ng/mL ^a			
		BoNT/A	BoNT/B	BoNT/E	BoNT/F
0-4	Negative	<2	<4	<2	<4
5-8	Weak Positive	2-5	4-10	2-5	4-10
9-21	Positive	5-50	10-60	5-50	10-60
22-27	Strong Positive	50->80	60->100	50->80	60->100

a Toxins attained a maximum colour score at approximately 100 ng/mL concentration and further concentrations tested were not included in the table and figure 4

Table 2: Survival times observed in mice after intraperitoneal (i.p.) injection (0.5mL per 20g BW) of BoNTs A, B, E and F.

Sample	Animal number ^a	Dose (D) (ng/kg)	Survival time (T) (h) ^b	D/T (ng/kg h)
BoNT/A	1	1	57.00	0.018
	2	1.5	55.33	0.03
	3	2	51.33	0.04
	4	2.5	42.67	0.06
	5	3	29.33	0.10
	6	3.5	19.33	0.18
	7	4	12.50	0.32
	8	5	10.33	0.48
	9	10	9.62	1.04
BoNT/B	1	1	68.67	0.015
	2	1.5	61.33	0.024
	3	2	52.67	0.038
	4	2.5	47.67	0.052
	5	3	41.33	0.073
	6	3.5	26.66	0.131
	7	4	13.83	0.289
	8	5	10.76	0.464
	9	10	9.00	1.11
BoNT/E	1	1	64.66	0.015
	2	1.5	60.67	0.025
	3	2	28.67	0.07
	4	2.5	23.30	0.107
	5	3	18.43	0.163
	6	3.5	13.00	0.269
	7	4	11.33	0.353
	8	5	10.00	0.50
	9	10	9.33	1.07
BoNT/F	1	1	88.66	0.011
	2	1.5	86.00	0.017
	3	2	83.33	0.024
	4	2.5	77.00	0.032
	5	3	71.33	0.042
	6	3.5	63.33	0.055
	7	4	59.33	0.067
	8	5	39.33	0.127
	9	10	9.33	1.07

a represent a group of three mice**b** Mean of three mice scores

Table 3: Results of the optical immunoassay (OIA) and the mouse bioassay to detect purified BoNT/A in spiked food samples.

BoNT/A concentration (ng/mL)	Orange juice		Buffer	
	OIA ^a	Mouse bioassay	OIA ^a	Mouse bioassay
20	13.67	+	10.33	+
4	3.33	+	5.67	+
0.8	1.33	+	2.67	+
0.16	0	+	0.67	+
0.032	0	-	0	+

(a) Values are mean of triplicate colour scores. An OIA score of ≥ 5 was considered positive; **(+)** non-protected mouse died or showed signs of botulism and the antibody protected mouse remained healthy; **(-)** both mice survived and non-protected mouse showed no signs of botulism

FIGURES

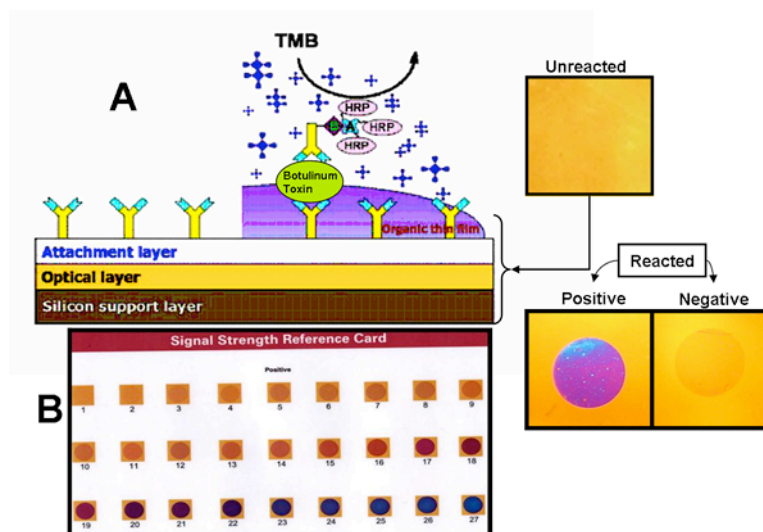


Figure 1: OIA principle for botulinum toxin detection: (A) The silicon wafer comprises of three layers viz., silicon (Si>100nm thick) support layer, an antireflective optical layer (silicon nitride, Si₃N₄ 47.5nm thick), and the attachment layer (aminoalkyl functional T-structure polymer, polydimethylsiloxane (TSPS)/poly (phenylalanine-lysine), PPL 13.5nm thick) that enhances antibody binding. The assay was based on the detection of physical changes on the thickness of molecular film resulting from immunobinding events on top of optical surface. Change in colour of reflected light from gold (unreacted) to purple blue (reacted) depends on the thickness of the molecular film formed and scored from the signal strength chart (B).

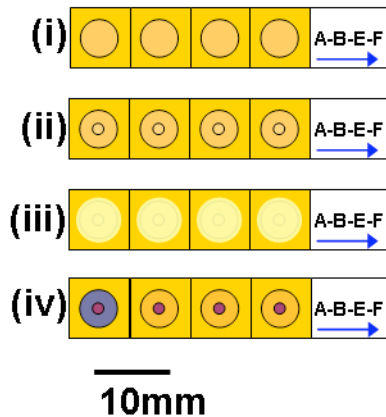


Figure 2: OIA test chip: Schematic representation of the salient steps involved in the preparation and detection of toxins (i) chips mounted on polypropylene support were coated with toxin specific capture antibodies to the four BoNTs A, B, E and F, followed by centre spotting of internal positive controls (ii) and blocking. Test samples were reacted to the test strip (iii), and results were read after formation of immunocomplex with HRP enzyme through avidin-biotin interaction and precipitation of TMB substrate(iv). The spot colours were scored for detection (positive/negative) and semiquantitative determination of the toxins.

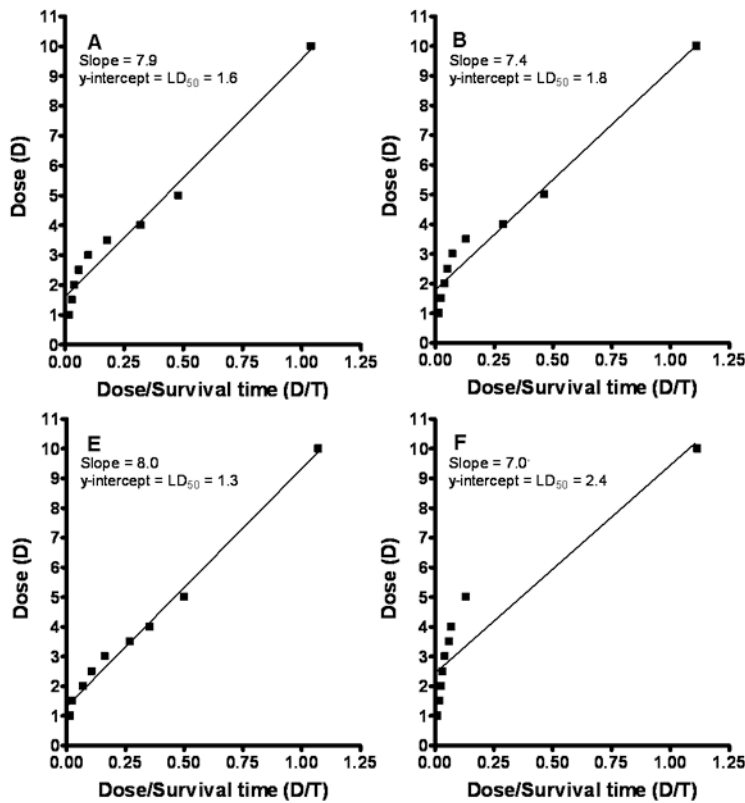


Figure 3: Plot of dose/ survival time (D/T in ng/kg.h, Abscissa) versus dose (D in ng/kg, Ordinate) of LD₅₀ determination experiment for botulinum neurotoxins BoNT/A (A), BoNT/B (B), BoNT/E (E) and BoNT/F (F). The y-intercept where the regression line intersects the ordinate is the smallest dose that kills 50% of the mice in an unlimited time and represents an approximate LD₅₀.(in ng/kg.h)

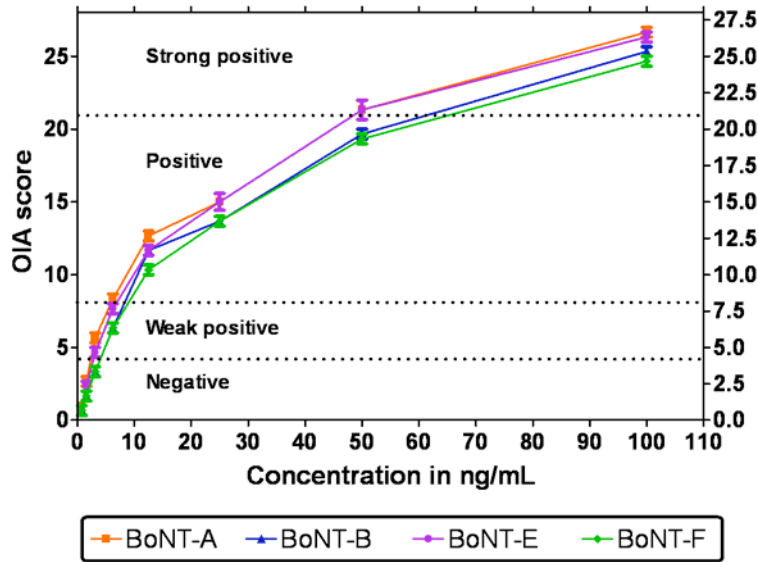


Figure 4: Sensitivity of OIA in the detection of BoNTs A, B, E, and F: BoNTs were double diluted in assay buffer and tested with specific antibody coated OIA. The assay was done in triplicate as described in materials and methods. OIA result was read and scored according to the colour score chart from 1 to 27. The values of OIA are plotted as mean \pm 1SD. The cut-off positive score for OIA was determined to be 5. The dotted lines classify the semi-quantitation as stated in table 1.

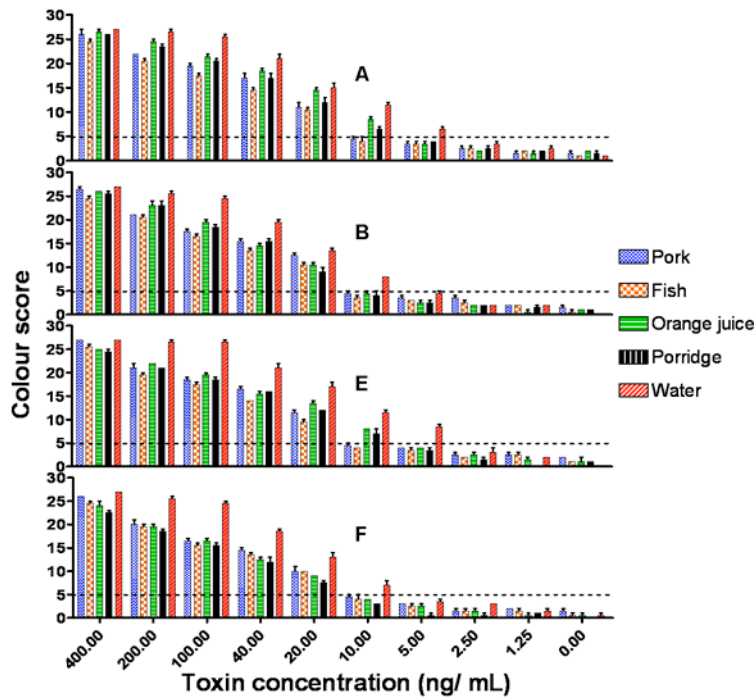


Figure 5: Graphic presentation of OIA colour scores obtained for the detection of BoNTs A, B, E and F in spiked food matrices (pork, fish, orange juice, porridge, and bottled water) using a concentration range from zero to 400ng/mL. Values represent the mean and S.D. of the triplicate test spots. The dashed line shows the OIA cut-off colour score of 5.

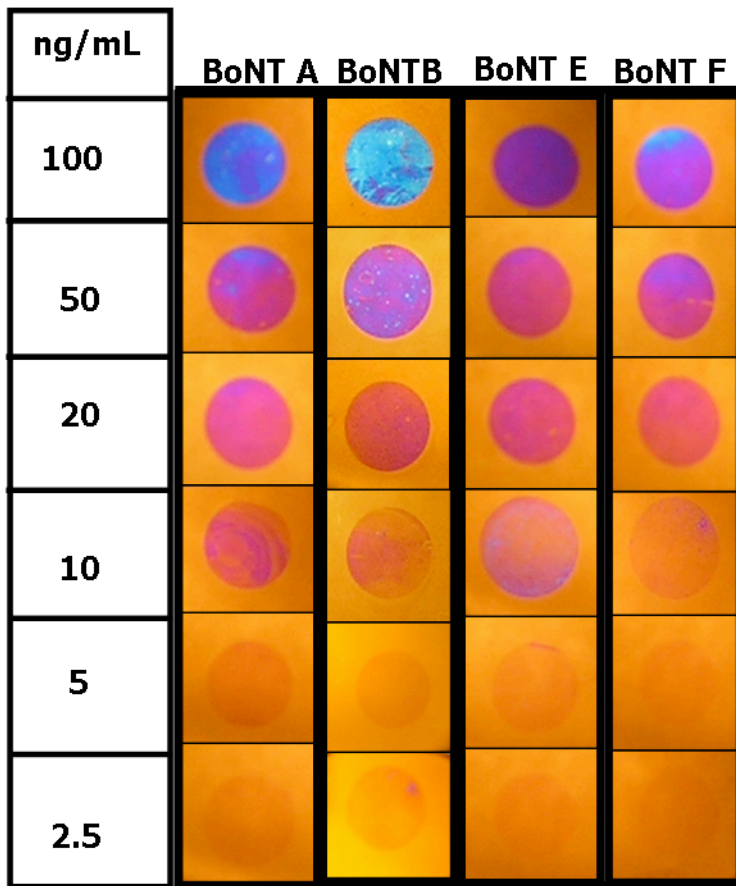


Figure 6: Limit of detection (LOD) for BoNTs A, B, E, and F in orange juice was represented for a concentration gradient (100 to 2.5 ng/ mL) of BoNTs. The LOD value was found to be ~10ng/mL for toxins A and E, and ~20ng/mL for toxins B and F.

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