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Rapid detection of botulinum neurotoxins A, B, E, and F by optical immunoassay

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1. ABSTRACT

Botulism is caused by the neurotoxins (BoNTs) produced from *Clostridium botulinum.* These neurotxins often lead to fatal neuroparalytic disease which is regarded a major threat to the public health. For this reason, rapid and reliable diagnosis of BoNTs in field settings and peripheral care centers is highly valuable. Here, we describe a multiplexed and sensitive optical immunoassay (OIA) for the rapid detection of four medically important BoNTs (A, B, E, and F). The assay is based on detection of physical changes in the thickness of molecular thin film resulting from specific immunobinding events on an optical silicon chip. The immunocomplex causes destructive interference of a particular wavelength of reflected white light from gold to purple-blue on an optical surface depending on the concentration of the analyte. This test allows simultaneous characterization of toxin type and semi-quantitative assessment of toxin level. The assay was four times more sensitive than ELISA when performed with the same reagents. The limit of detection (LOD) for the BoNTs was, respectively, 2.5-5 ng/mL, 5-10 ng/mL, and 10-20 ng/mL in experimentally spiked buffer, water and food matrices. The less logistic load and visual read-out of this method promises potential applicability of this assay in the field as well as in a clinical settings.

2. INTRODUCTION

Botulism is caused by the neurotoxins (BoNTs) produced from *Clostridium botulinum.* There are seven antigenically distinct (A to G) but structurally similar types of BoNTs which fall into genotypically and phenotypically distinct groups (I to IV). Human botulism is often times associated with Groups I and II which include toxinotypes A, B, E, and F (1-5). The potential of these neurotoxins is known in causing food poisoning and in use as biological warfare agents. The median lethal dose of BoNT was estimated to be 0.3 ng/kg through intravenous route, 20 ng/min/m^3 upon inhalation, and 1μg/kg after ingestion (6-7).

Preventive measures and rapid method for analysis of food, air and water supply deliberately contaminated by botulinum toxin are of great interest. A significant progress has been made over the past decade in delineating the mode of action and development of novel detection methods for these neurotoxins. Mouse lethality assay has remained the gold standard test for the detection of BoNTs, in view of its exquisite sensitivity with a reported detection limit of 1 mouse LD_{50}/ml (1 mLD_{50} = 10-20 pg of BoNT/ml). However, this test has a number of drawbacks including a large number of animal required, expense, inability to

Table 1. Assays in the detection of botulinum neurotoxins

Type	Assay time	Sample	LOD[1]	Reference
Passive hemagglutination	5-6h	Serum BoNT/A/B/E	1.3.-1.6. mLD_{50}	33
Radio immunoassay	8h	BoNT/A purified	100 mLD_{50}	34
Mouse bioassay	3-4days	BoNT/A	1-2 mLD_{50}	35
ELCA	>18h	Culture supernates	<1 mLD_{50}	36
Protease activity assay-fluorescence	3h		4pM	37
Time-resolved fluorescence	>2h	BoNT A/B in Spiked buffer	4-20pg/ml	38
Amplified ELISA	8h	Chilli, potato	0.2. – 1ng/ml	39, 40
Ganglioside-liposome immunoassay	20 min	Buffer	100pg/ml (visual), 15pg/ml (densitometry) = 3 mLD_{50}	41
Immuno-PCR				42
Protease activity assay-MS	4 hr	BoNT-A/B/E/F	0.0.7 pM, 1.2.MLD_{50}/ml (A/B) 6.2.MLD/ml (E)	43
Endopep-MS	4 h	BoNT-A/B/E/F in spiked serum	20 mLD_{50} /ml (A), 1 mLD_{50} (B), 0.2. mLD_{50} (E), 1mLD_{50} (F)	44
ECL BoNT A,B,E, F	2 hr	BoNT-A/B/E/F in spiked Water, Serum, urine, buffer , milk, meat, egg, apple juice	0.0.5 – 0.8.ng/ml	9
DIG ELISA	4 hr 15 min	Casein buffer Food sample	60.3.pg/ml (A),176pg/ml (B), 163pg/ml (E), 117pg/ml (F) 2ng/ml	1
Plastic ELISA-on-a-chip (EOC) biosensor	20 min	Purified BoNT/A in Spiked buffer	2ng/ml	3
Vertical-flow strip immunochromatography (ICT)	40 min		20 to 40 pM (220 to 440 MLD_{50}/ml)	45
disposable immunoaffinity column (IAC)	40 min	BoNT/A complex Assay buffer Milk Serum	0.4.5 pM (5MLD_{50}/ml) 1.8.2pM 0.0.9pM	

[1]1 mouse LD_{50} /ml is ~ equivalent to 10-20pg/ml or ~70-140fM

discriminate the neurotoxin type unless additional neutralization tests are carried out in parallel and the 4 day requirement for obtaining test results,. A number of immunoassays in variable formats and molecular methods overcoming these drawbacks with equivalent or comparable sensitivities have been developed (Table 1) (8). Although, more recently, electrochemiluminescence (ECL) based detection of botulinum toxins with a sensitivity greater than ELISA has been demonstrated, the test is time consuming, more expensive, less robust, and has a lower reproducibility (9-10). Since ECL has to be performed in a laboratory, this test can not be used in a field setting. Other, available smart tickets available in the first responder detection kits are relatively simple and rapid but such tests are qualitative, have variable sensitivity or lack the ability to discriminate the toxin types (11-13). For these reasons, there is a need for rapid, sensitive and reliable assays for the detection of BoNTs in field and clinical settings. To overcome the disadvantages of existing immunoassay formats, an optical immunoassay (OIA) can serve as a better alternative to these tests. The assay is simple and user friendly, and is based on the detection of changes in the reflected white light from a silicon optical coated surface. The immunobinding events on the surface of the optical chip cause destructive interference of a particular wavelength of reflected light 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 can be easily interpreted by naked-eye and require less logistic load to perform in the field. There have been a number of studies that employed OIA platform for the diagnosis of infectious diseases, brain trauma markers, monitoring of purified proteins, and in the detection of snake venom toxins (14-22).

The present study reports the application of OIA for the multiplexed determination of BoNTs (A, B, E, and F) in different matrices. The test was constructed as an antibody coated optical strip assay with built-in positive control. Toxin was identified based on the position of the positive spot as specified on the strip. Simultaneous semi-quantitative estimation was carried out based on the color scores coded on a chart. The ease of use and less turn-round time for diagnosis by OIA would allow on-site detection of suspected cases.

3. MATERIALS AND METHODS

3.1. Optical surface assay system

The silicon assay system (SILAS™-I) was obtained from ThermoBioStar, Colorado, USA. The kit was comprised of silicon optical reflecting surface for protein immobilization; blocking reagents (Blockaid™); tetramethylbenzidine (TMB) substrate solution pH 5.5. containing hydrogen peroxide; and signal strength chart, which scores the full reflection color spectrum of this optical surface from 1 to 27 according to the thickness of the layer formed on top of it (Figure 1B). ELISA plates were purchased from Nunclon (Denmark), soluble TMB substrate from Dakopatts, USA.

3.2. Toxins and antibodies

Purified BoNTs were procured from Metabiologics Inc., USA. Polyclonal antibodies (PAbs) to BoNTs were obtained from Statens Serum Institute, Denmark. Biotin N-hydroxysuccinimide ester and dimethyl

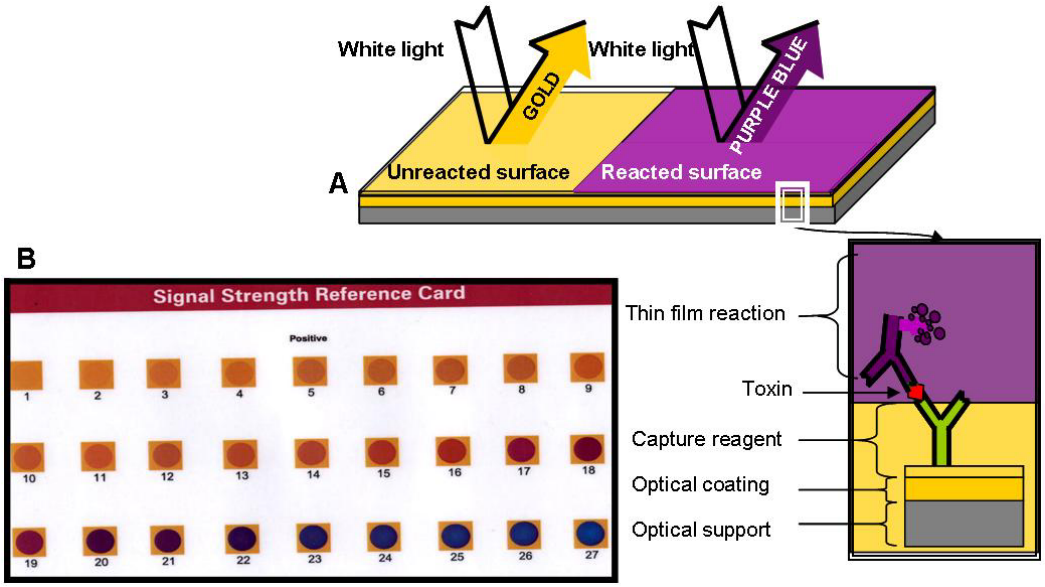

Figure 1. OIA Principles of optical immunoassay for snake venom detection. (A) The assay was done based on the detection of physical change in the thickness of molecular thin films resulting from specific binding events on the top of an optical silicon surface. The silicon wafer comprises three layers: (i) silicon supporting layer, (ii) an optical layer which reflects the light (iii) and the attachment layer on the top which enhances the binding of capture antibody. Formation of immunocomplex with HRP enzyme attached via avidin-biotin interaction and the precipitation of TMB substrate produce a biological thin layer on the optical reflecting surface. Presence of the thin film changes the reflected light from gold colour into purple or blue depend on the thickness of the optical layer or the amount of immunocomplex formed. (B) Signal strength chart, which scores the full reflection colour spectrum of this optical surface from 1 to 27 according to the thickness of the layer formed on top of it.

sulfoxide (DMSO) from Sigma, Econo-Pac Protein-A affinity and desalting columns from Bio-Rad, USA. Proteins 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.

3.2.1. Purification and biotinylation of IgG

The steps involved included affinity chromatography of IgG from hyper-immunized rabbit sera with protein A columns, and immuno-absorption of cross-reactive antibody molecules with heterologous toxin columns. The resulting antibodies were toxin-specific as tested by ELISA. The toxin specific PAbs were biotinylated as described with minor modification (23): Briefly the method consisted of the following steps: 0.2.ml of Biotin-N-hydroxysuccinimide ester (2mg/ml in DMSO) was mixed with 1 mg/ml aliquot of antibodies incubated for 4 hr at room temperature. The mixture was then centrifuged at 5000 rpm for 10 min and the supernatant dialyzed against PBS pH 7.4. with two changes of buffer using a centrifuge filter device (Amicon Ultra-15, Millipore, Ireland). Aliquots of biotinylated antibodies were stored at −80 °C until use.

3.3. Preparation of OIA strips

Capture antibodies (2.5.µg ml^{-1}) in PBS pH 7.4. were manually spotted in an orderly pattern on each chip of a 4-chip test strip mounted on a plastic holder using double sided tape. Each spot contained 30 microliters of sample to form a spot about 5 mm in diameter. The spotted strip was then incubated overnight in a humid chamber at 4 °C. The strip was washed with washing buffer (PBS, pH 7.4., containing 0.0.5% Tween-20 (PBS-T20)) and the unoccupied sites were then blocked by incubating in blocking buffer (1% Blockaid™ diluted in PBS, pH 7.4. containing 0.5.% T20) for four hours at 4 °C. After thorough rinsing with washing buffer, a 1.5.µl of internal positive control (mixture of four toxins to 25ng/ml each) was center spotted on each chip of the strip about 2mm in diameter. Similar washing and blocking steps were followed and the strip was then air-dried and stored at 4 °C until use. Unused reactive surface were used as negative control in the assay.

3.4. Sensitivity assay

3.4.1. OIA procedure

The test samples (BoNTs of concentrations from 0-40ng/ml in spiked buffer) (50 µl) were placed on the centre of the chip to cover the whole chip and incubated for 10 min. After rinsing with washing buffer from a squeeze plastic bottle for 40 s and drying with air from a squeeze rubber bulb for 20 s, pooled biotinylated antibody solution at 2 µg ml (30 µl) was added and the chip was incubated for 10 min. After another washing and drying cycle, HRP-avidin conjugate (30 µl) was dispensed onto each chip and incubated for 5 min. The chip was washed, dried again and substrate solution (25 µl) was applied onto the centre of the chip and incubated for 5 min. The entire procedure was

performed at room temperature. Color changes were observed after the final washing with deionized water and drying by naked eye under a white light source. The results were recorded by the changes of color of the spots against the gold colored background. The position of the positive spot indicates the toxin type detected in the test sample. The amount of toxin is semi-quantitated by referring to color strength score chart. The sample dilutions were run in triplicate.

3.4.2. Analysis of BoNTs in spiked matrices by OIA

Spiked matrices include liquid food samples such as bottled water (100ng/ml) and Orange juice (100ng/ml) of purified toxins A or E, or 200ng/ml of B or F. A 5ml sample was incubated for 30min at room temperature followed by centrifugation at 4000 × g (Kubota Corporation, Tokyo) for 20min at 10^{o}C to remove solid particles. Supernatant was diluted 1:1 ratio in PBS and used for the assay. For semisolid food samples such as porridge, 10g of food was spiked with 200ng/ml of BoNT A or E, or 400ng/ml of B or F. Processed as above except for a brief vortex step before and after incubation.

3.4.3. ELISA procedure

Double antibody sandwich ELISA was constructed and performed with the same reagents for each of the toxin type individually run in triplicates, with plain buffer as negative control. The toxin samples were added into the corresponding antibody coated wells and incubated at room temperature for 1h. After 5 washings, biotinylated antibodies (100µl) were added into the wells and incubated for another 1h at room temperature. Following another washing cycle, 100µl of avidin-HRP conjugate was added into each well and incubated for 30 min. Finally the wells were washed and 100µl of TMB substrate solution was delivered into each well and incubated for 5 min then the color development was terminated by the addition of 50µl of stop solution (2N sulphuric acid) to each well. The optical density (OD) was measured at 450nm with E max microplate reader.

3.4.4. Statistical analysis

ELISA and OIA analysis of BoNTs A, B, E, and F was performed in triplicates for sensitivity determination. Statistical analysis was performed with GraphPad Prism software version 2.0., GraphPad Software Incorporated, 1995.

4. RESULTS AND DISCUSSION

In the present study, enzyme immunoassay was carried out on an optically active silicon surface (SILAS™-I), which was specially designed for protein immobilization. The OIA surface technology enabled direct visual detection of a physical change produced by specific antigen-antibody interactions.

4.1. Optimization of OIA and ELISA

All anti-BoNT (A, B, E, and F) pairs were evaluated first by ELISA to determine optimum antibody pairs for use in a capture ELISA. The biotinylated antibodies were evaluated by standard checkerboard titrations. The optimal capture and detector antibody concentrations providing the best signal to noise ratio were used in all subsequent experiments. The parameters which were optimized included coating and detector antibody and blockaid concentrations, incubation time, buffer. Similarly, OIA was independently optimized for the preparation of coated strips, positive control spot, and incubation time. The least incubation time that gave reproducible result was followed in all subsequent analysis.

4.1.1. Cross reactivity

Cross reactivity at 20 ng/ml of BoNTs was evaluated with each type specific antibody. The affinity purified antibodies used in these assays demonstrated with <1% cross reactivity between corresponding toxin and other toxin types (data not shown). Although, BoNT serotypes exhibit up to 60% sequence homology, prior studies with serotype specific antibody reported little or no cross-reactivity (24-26). Conversely, significant cross-reactivity of toxin A with B antitoxin and vice-versa was observed in some studies, however, this reactivity was minimized by absorption with heterologous antigens without affecting the titer towards homologous antigens (27-28).

4.2. Detection of toxins in assay buffer and food matrices

4.2.1. Sensitivity of OIA

The sensitivity of the OIA was assessed in spiked assay buffer and the limit of detection (LOD) was found to be 2.5 ng/ml (78.8. LD_{50}) for BoNT type A (BoNT/A) and BoNT/E (69 LD_{50}), 5 ng/ml for BoNT/B (44.9. LD_{50}) and BoNT/F (34.2. LD_{50}). Comparison of OIA with ELISA revealed elevated sensitivity (4 times) of OIA performed with the same set of reagents (Figure 2).

4.2.2. Detection of BoNTs in food matrices

Standard curves for BoNTs A, B, E and F were constructed from OIA data derived from spiked water, orange juice and porridge (Figures 3A-3C). A minor variation in the detection limit was observed with different matrices. Data for toxin types in spiked water are shown in Figure 3A. The assay effectively measured 5 ng/ml (150 pg/spot) for BoNTs A/E and 10 ng/ml (300 pg/spot) for BoNTs B/F. Analysis of spiked orange juice and porridge were presented in Figures 3B and 3C. The LOD was relatively less compared to that obtained with assay buffer with 10ng/ml (300 pg/spot) for BoNTs A/E and 20ng/ml (600 pg/spot) for BoNTs B/F. Although sensitivity achieved by OIA in our study is well below the estimated lethal dose of BoNTs for humans by any route as specified for a 70 kg body weight would be 0.0.9-0.1 5µg intravenous or intramuscular, 0.7.-0.9 µg through respiratory, or 70 µg by ingestion (29). However, it was possible to improve the sensitivity of OIA by virtue of fieldable sample processing protocols that utilize immunoaffinity columns. It is perhaps possible to extend the use of this system for other bio-toxins and infectious agents.

Multiplexed immunoassays for the detection of more than one analyte in a single test have gained much

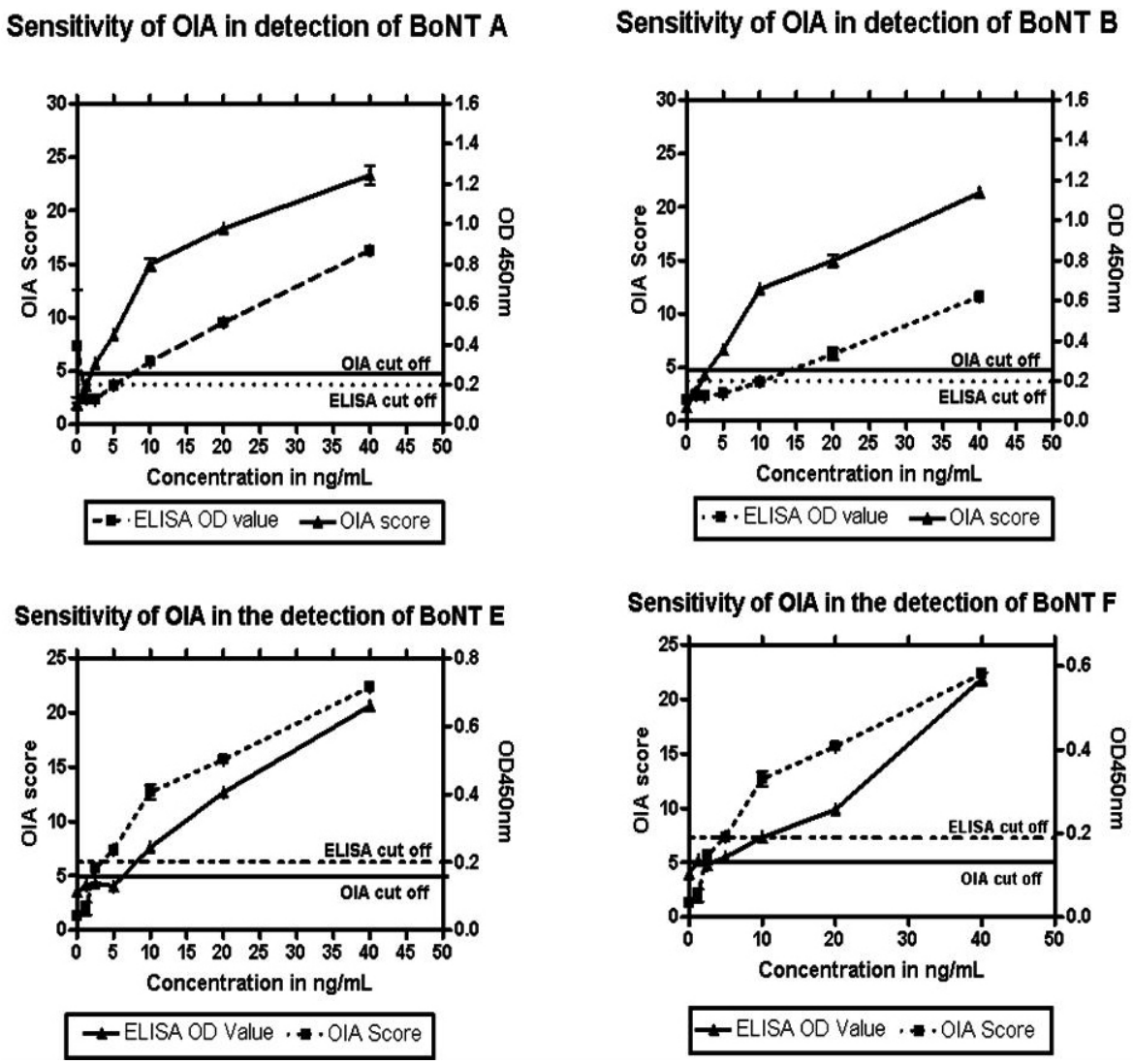

Figure 2. Sensitivity assay of OIA in the detection of BoNTs A, B, E, and F spiked in assay buffer. The values of OIA and ELISA are plotted as mean plus or minus 1standard deviation of the triplicate scores for OIA and OD values for ELISA. The cut-off for ELISA was determined to be 0.2. and that of OIA with a score of 5. The overall sensitivity of OIA has revealed 4 times more than a standard ELISA.

recognition (30-32). In the present study, a four analyte immunoassay was presented for the detection of neurotoxins of *Clostridium botulinum.*. OIA provided a direct "yes-or-no" result suitable for efficient identification of the four BoNTs based on the distinct positive spot position on the assay strip (Figure 4A-C). The test could simultaneously determine the toxin type and concentration based on the color strength with 30 µl of analyte sample per chip (120 µl per strip) that is less than 3 times the volume required for ELISA in a single test. In addition, the test was less expensive ($28 per assay strip).

The length of the assay remains a limiting factor for carrying out the test in field. Although decreasing this time frame would be highly desirable as a 'point-of-care' test, shortening the incubation time could affect the sensitivity of the assay. To overcome this inherent problem, some studies adopted combined incubation of antigen and capture antibody, included different species of capture and detector antibodies or used monoclonal antibodies (21, 31-32). The capture and detector type specific antibodies used in this study were from the same animal and this limited their combined use (as may compete with capture antibodies for binding sites on antigens). Nonetheless, we found the OIA to be substantially rapid (assay duration of 35 min) and more sensitive than standard ELISA for the detection of BoNTs. In conclusion, the present OIA provides an invaluable screening tool for the detection of BoNTs in a variety of food. This assay overcomes the limitations of existing LFAs, a need for expensive instrumentation, or laboratory dependence. Other advantages include visual read-out in the field, as well as reduced logistic load without the need for a power source, or multiple analyte measurements.

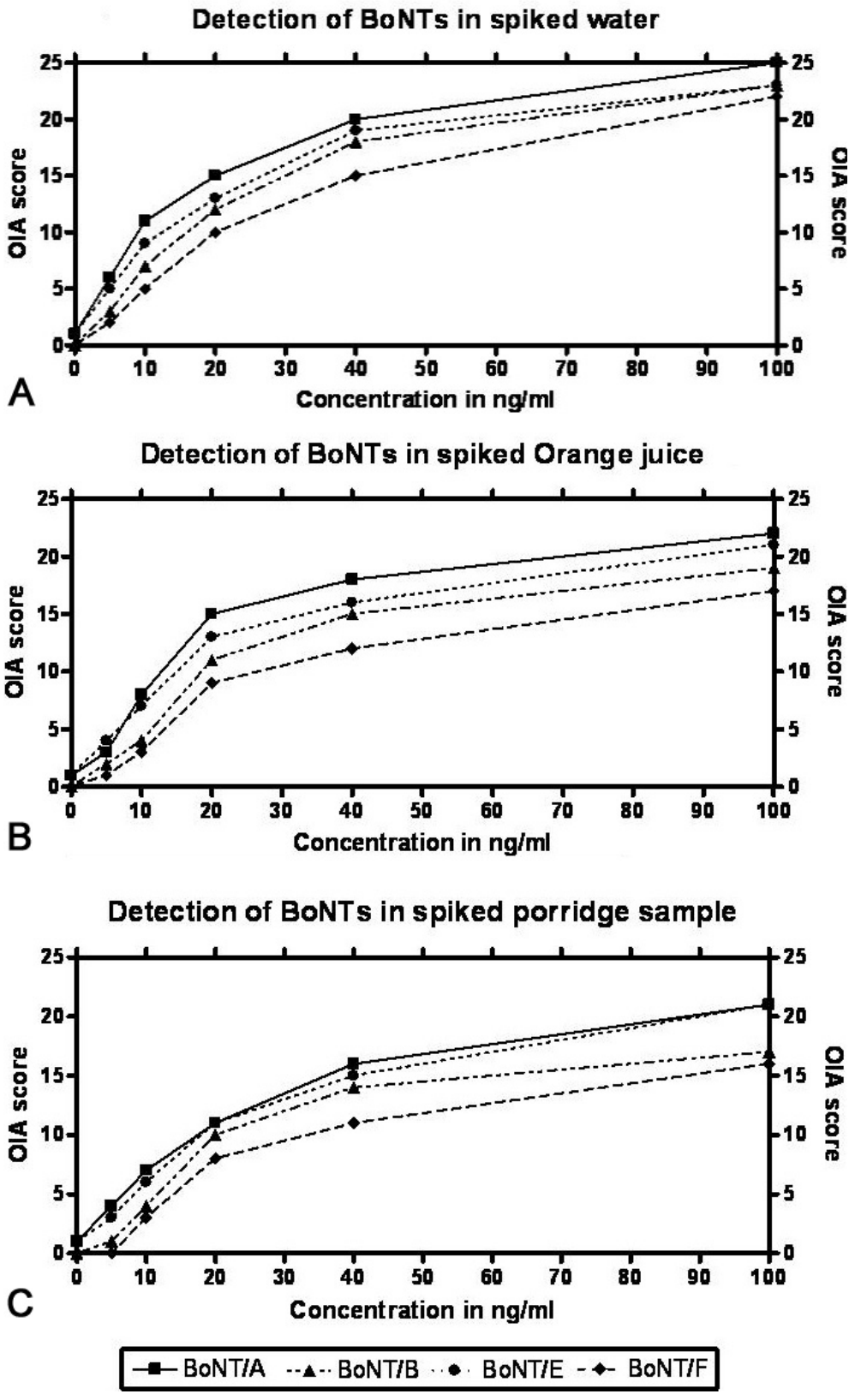

Figure 3. Figure depicts the detection limit achieved by OIA (strip assay) in the detection of BoNTs A, B, E, and F in selected food matrices. The concentrations represented the actual spiked values per milliliter of matrix.

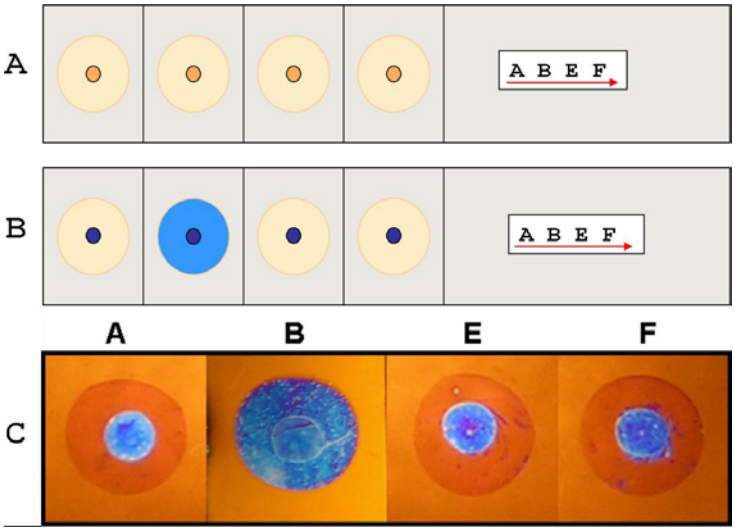

Figure 4. Schematic representation of customized assay strip (A) unreacted (B) showing positive reaction for BoNT/B (C) Original reacted strip in multiplexed assay showing a central blue spot of internal positive controls and a positive spot development in the position specified for BoNT/B on the assay strip.

5. ACKNOWLEDGEMENT

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Abbreviations: BoNTs: bolulinum neurotoxins; DIG: digoxigenin-label; DMSO: dimethyl sulfoxide; ELISA: enzyme linked immunosorbent assay; ECL: electrochemiluminescence; ELCA: enzyme linked coagulation assay; HRP: horse-radish peroxidase enzyme; LD_{50}: lethal dose 50; MS: mass spectrometry; PBS: phosphate buffered saline; PAbs: polyclonal antibodies; TMB: 3.3.', 5.5.'-tetramethylbenzidine

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