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Research paper

Rapid and selective detection of botulinum neurotoxin serotype-A and -B with a single immunochromatographic test strip

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ABSTRACT

Botulinum neurotoxins (BoNT) are the most potent toxins known. Produced by *Clostridium botulinum*, BoNTs are classified into seven, antigenically distinct serotypes, designated A–G. The toxin acts to inhibit acetylcholine release, resulting in paralysis and death. Naturally occurring foodborne disease is most often the result of improper canning of foods, while wound botulism, associated with injection drug users, is on the rise. Because of its potency, BoNTs have also been identified as targets for use by bioterrorists. The 'gold standard' of detection of BoNTs is the mouse bioassay, an expensive and time consuming test that requires specialized equipment and trained personnel. There is a need for a rapid, sensitive diagnostic for BoNTs that could be used by minimally trained personnel in the event of a foodborne outbreak or a bioterrorist threat. Here, we describe the use of a single lateral flow device (LFD) that can detect and distinguish between BoNT/A and B, two of the four serotypes that are known to intoxicate humans and together represent >80% of naturally occurring illness. The device could detect as little as 5 ng/mL of purified BoNT/A and 10 ng/mL of BoNT/B in 2% and 1% milk, respectively. In undiluted apple juice, 25 ng/mL of BoNT/A and 10 ng/mL of BoNT/B could be detected. No cross reactivity between BoNT/A and B antibodies was observed. The LFD described here is easy to use, requires no specialized training or equipment, and can identify and distinguish between BoNT/A and /B serotypes. These attributes make this rapid diagnostic device a potentially valuable tool in the fields of food safety and homeland security.

1. Introduction

Clostridium botulinum is a spore-forming obligate anaerobe which occurs naturally in the soil and is the causative agent of foodborne, wound and infant botulism (Shukla and Sharma, 2005). Germinating spores of distinct strains of *C. botulinum* produce and secrete different serotypes of botulinum neurotoxin (BoNT), designated A–G, which can be absorbed through mucosal surfaces (Swaminathan, 2011). Aerosolization of BoNT as a means of dissemination can pose a bioterrorist threat (Shukla and Sharma, 2005; Arnon et al., 2001; Eubanks et al., 2007).

BoNT is produced as a dichain polypeptide that is then cleaved into a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC) (Montal, 2010). While the HC facilitates entry of the toxin into neurons by endocytosis, the LC is a metallopeptidase that cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), inhibiting acetylcholine release and resulting in flaccid muscle paralysis (Montal, 2010; Schiavo et al., 2000). In humans, a lethal dose intravenously or intramuscularly is estimated at 1–2 ng/kg body weight; orally at 1 μg/kg and 10–12 ng/kg by inhalation (Arnon et al., 2001).

Given their potency, BoNTs have been employed as therapeutics, as tools in basic science research, and as weapons of biological warfare (Arnon et al., 2001; Shukla and Sharma, 2005). The gold standard of detection of BoNTs

Abbreviations: BoNT, botulinum neurotoxin;LFD, lateral flow device;HC, heavy chain;LC, light chain;mAb, monoclonal antibody;ELISA, enzyme-linked immunosorbent assay.

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is the mouse bioassay, which can detect as little as 10 pg/mL of toxin (Sharma and Whiting, 2005). However, the assay requires several days to complete, large numbers of animals and can only be performed at a select number of laboratories in the United States. To determine the serotype, a second, independent neutralization assay is required. In the event of a suspected BoNT contamination event, the mouse bioassay, while extremely sensitive, does not meet the needs of emergency responders. Therefore a rapid, sensitive, and selective BoNT diagnostic test that can be field deployed could be used to address suspect BoNT contamination.

A number of *in vitro* assays to detect BoNTs, including ELISA kits, PCR-based methods and assays based on the enzymatic activity of the toxin's light chain have been developed (Chao et al., 2004; Wictome et al., 1999; Shone et al., 1985). While some of these methods have comparable sensitivity to the mouse bioassay, they still require trained personnel and specialized equipment. In contrast, lateral flow devices (LFDs) are simple, low cost alternatives that can be easily deployed in the field and do not require specialized training to operate or to interpret the results. LFDs can be read without optical detection systems, are compact, and on average have a long shelf life (Posthuma-Trumpie et al., 2009; Warsinke, 2009; Ngom et al., 2010). While these devices typically have less sensitivity than ELISA formats, they do offer a method for rapid, simple assessment of potential BoNT contamination to a multitude of personnel.

Our laboratory has developed several high affinity monoclonal antibodies (mAbs) that selectively recognize the BoNT/A and /B serotypes. MAb F1-2, which recognizes the N-terminus of the heavy chain of BoNT/A, has been extensively characterized and effectively employed as a capture antibody in a sandwich ELISA (Scotcher et al., 2009; Stanker et al., 2008). We have also previously described MCS-6-27, a BoNT/B-specific mAb that binds the carboxyl portion of the HC and can be used as a capture antibody in a sandwich ELISA (Scotcher et al., 2010). Both these mAbs are highly specific for their respective serotypes. Here, we describe the use of F1-2 and MCS-6-27 capture antibodies in combination with two novel detection antibodies developed in our laboratory, F1-51, a BoNT/A HC-specific mAb and BoB-92-32, a BoNT/B HC-specific MAb, in the development of a rapid BoNT LFD.

Our LFD is capable of resolving BoNT/A and /B as two independent colorimetric lines on a single strip, with sensitivities >10 ng/mL for purified toxins and 10–500 ng/mL in toxin fortified beverages. These results demonstrate the capability of these mAb pairs to simultaneously detect BoNT serotypes A and B on a simple and inexpensive immunochromatographic test strip. These devices could be used to aid in BoNT detection by first responders or as part of commercial food processing where natural contamination of *C. botulinum* bacteria is suspect.

2. Materials and methods

2.1. Reagents and components

Botulinum toxins (BoNT) serotypes A and B where purchased from Metabiologics, Inc (Madison, WI). Colloidal gold (40 nm), PVC backing cards and plastic cassettes were purchased from Diagnostic Consulting Network (Carlsbad, CA). Immunopore SP membrane, CF6 absorbent sink, Standard14 conjugate release pad and Fusion5 membrane were purchased from GE Healthcare. Affinity purified donkey anti-mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA).

2.2. Monoclonal antibody production and purification

The monoclonal antibodies F1-51 and BoB-92-32 were produced as previously described (Scotcher et al., 2010; Stanker et al., 2008). F1-51 was demonstrated to bind the HC of BoNT/A while BoB-92-32 bound the HC of BoNT/B (unpublished observation, LHS).

2.3. Synthesis of colloidal gold conjugated probe

The lowest possible concentration of mAb required for stabilizing colloidal gold particles was prepared according to previously published procedures with some modification (Yokota, 2010). Briefly, each mAb was diluted to 5, 15, 20, 25, 30 and 40 μg/mL in water and the pH was adjusted to 9 with 0.2 M K_2CO_3. Next, 0.5 mL of colloidal gold (pH 9) was added to 100 μL of each antibody dilution and incubated for 10 min at room temperature. Next, 100 μL of 10% NaCl was added to each tube and the change in color was assessed. The lowest antibody concentration with no color change represented the optimal concentration for stabilizing the gold sol. Antibody-gold conjugates were prepared using the determined antibody concentration. Unconjugated antibody was removed by centrifugation at 15,000 $\times g$ at 4 °C for 30 min. Conjugates were stored in buffer A (50 mM phosphate, pH 9, 0.1% tween-20, 1% BSA) at 4 °C.

2.4. Assembly of lateral flow device

Capture antibodies were diluted in 10 mM phosphate buffer with 3% v/v methanol and applied to the membrane at 1 mg/mL using a BioJet Quanti Dispenser (BioDot, Irvine, CA), dried at 37 °C for 30 min, then blocked in 10 mM PBS, 0.1% fish gelatin, 1% BSA and 0.5% Triton X-100 for 1 h. The blocked membrane was dried for 30 min at 37 °C and assembled on to the backing card with a 2 mm overlap by the absorbent sink. The conjugation pads were prepared by immersion of gold conjugated antibodies diluted in buffer containing 50 mM phosphate, 1% BSA, 5% sucrose and 0.1% tween-20. Conjugation pads were then dried for 30 min at 37 °C and fixed, with an ~2 mm overlap with the nitrocellulose, on the backing card. Finally, Fusion5 membrane was employed as the sample pad with an ~2 mm overlap with the conjugation pad. The entire assembly was housed in a plastic cassette (Fig. 1).

2.5. Preparation of BoNT-spiked beverages

Whole, 2% and 1% milk were obtained from a local grocery store. Apple juice and orange juice were obtained from a refrigerated vending machine. Spiked milk samples were assessed by two methods: (1) following a 10-fold dilution with double deionized water and (2) following centrifugation at 12,000 $\times g$ at 4 °C for 20 min to remove fatty content. Following spike with toxin, orange and apple juice were both

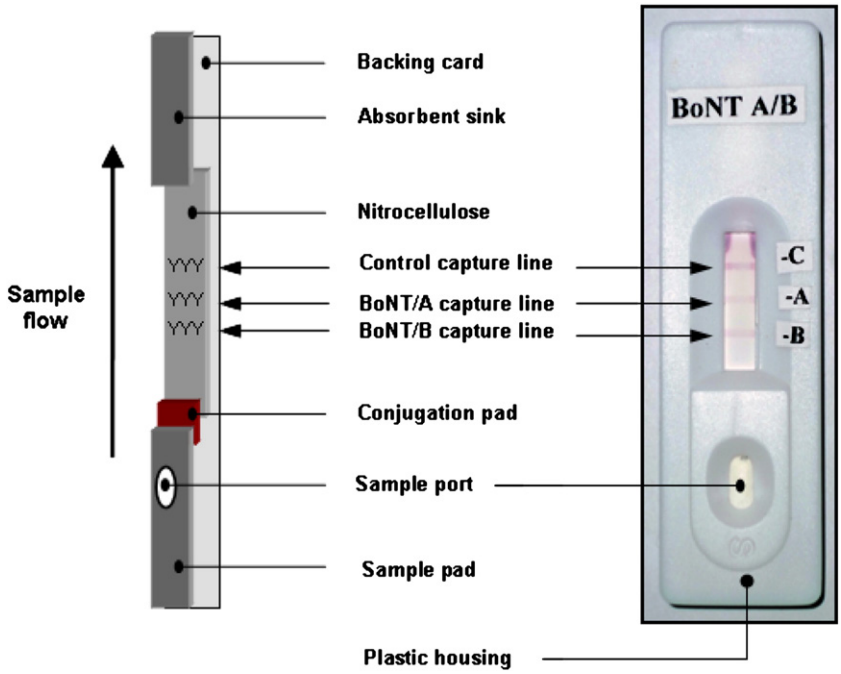

Fig. 1. Schematic of lateral flow device (LFD). The device consists of a nitrocellulose membrane to which an anti-mouse IgG (control capture line) and *C. botulinum* serotype specific capture antibodies (BoNT/A and BoNT/B capture line) are immobilized. Fixed to the lower end of the membrane is a conjugation pad containing immobilized anti-BoNT gold-conjugated antibody. A sample pad overlays the conjugation pad. At the upper end of the membrane is an absorbent sink which acts to facilitate capillary flow through the device. The entire device is fixed to a backing card and held in a plastic housing. Sample is added at the sample port, absorbed into the sample pad and flows by capillary action through the conjugation pad. If toxin is present it binds the gold-conjugated detector antibody, and together they flow to the test capture line where the immobilized capture antibody binds the antigen, resulting in the resolution of a red line obvious to visual inspection. The sample continues to flow over the control capture line which binds remaining gold conjugated detector antibody (either bound or unbound to toxin) resulting in a red line that validates proper test function. Remaining fluid and sample is absorbed by the absorbent sink at the end of the device. The actual LFD (right) shows an example of positive detection of BoNT/A and /B serotypes with the control line.

neutralized using 1 M NaOH. Orange juice samples were tested by two methods: (1) following a 2-fold dilution with phosphate buffer; and (2) following centrifugation to remove pulp. Apple juice samples were tested: (1) directly after spiking and; (2) following a 2-fold dilution with phosphate buffer.

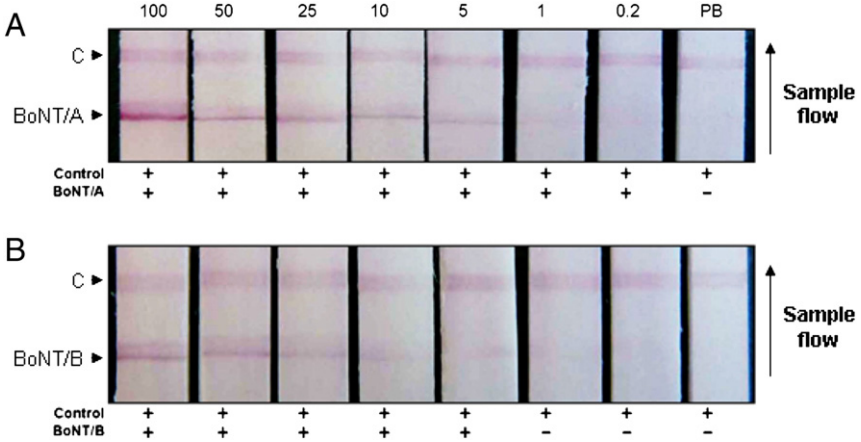

Fig. 2. Lateral flow detection of BoNT serotypes. A serial dilution (100 ng/mL to 0.2 ng/mL) of either BoNT/A or BoNT/B was prepared in phosphate buffer (PB). Fifty microliters of diluted BoNT/A or BoNT/B was then applied to lateral flow strips prepared with either BoNT/A-specific mAb F1-2 capture antibody (panel A) or BoNT/B-specific mAb MCS-6-27 capture antibody (panel B), respectively. The absence of a line in the test zone (BoNT/A or BoNT/B) following addition of phosphate buffer (PB) alone to the device served as control for non-specific binding. C is the location of anti-mouse control line. + indicates resolution of gold-conjugated antibody by immobilized control or test antibody; − indicates no resolution of gold-conjugated antibody by immobilized control or test antibody. Arrow indicates the direction of sample flow.

3. Results

3.1. Detection of BoNT/A and /B by lateral flow

Here, we investigated the application of mAb capture/detector pairs for BoNT/A and BoNT/B, developed previously in our laboratory, in a LFD. For the BoNT/A LFD, F1-2 and a control goat-anti mouse IgG were separately immobilized on a nitrocellulose membrane at 1 mg/mL using a BioJet Quanti Dispenser. The F1-51 mAb was conjugated to 40 nm gold particles and applied by immersion onto a conjugate release pad. A serial dilution of purified toxin, ranging from 100 to 0.2 ng/mL, was prepared in a phosphate buffer, and the assay was initiated by the application of diluted toxin (50 μL) to the sample pad (Fig. 2). A visible red line was resolved in ~10–15 min. As shown in Fig. 2A, detection of purified BoNT/A was easily visualized at concentrations of 100 to 5 ng/mL, and weakly visible at 1 and 0.2 ng/mL. No reactivity was observed when purified BoNT/B was applied at 100 ng/mL (data not shown) or with buffer alone. These results demonstrate the suitability of mAbs F1-2 and F1-51 for use in a sensitive and selective LFD to detect BoNT/A.

A BoNT/B LFD was also developed using mAbs developed in our laboratory. Anti-BoNT/B monoclonal antibody MCS-6-27 and control goat anti-mouse IgG were separately immobilized at 1 mg/mL on nitrocellulose, and mAb BoB-92-32 was employed as the detector antibody. A serial dilution, again ranging from 100 to 0.2 ng/mL of purified BoNT/B was evaluated. With a limit of detection of 5 ng/mL, the BoNT/B LFD was not as sensitive as the BoNT/A device (Fig. 2B). Increasing the concentration of the immobilized capture antibody did not improve the sensitivity of the assay (data not shown), however the BoNT/B LFD demonstrated high specificity, showing no reactivity with BoNT/A toxin (data not shown).

3.2. A single immunochromatographic strip to detect BoNT/A and /B

As individual assays, the monoclonal antibody pairs for BoNT/A and /B demonstrated high specificity for their respective serotypes. As such, we sought to determine if they could be used together in a single LFD capable of detecting both BoNT/A and /B. Control goat anti-mouse antiserum, mAbs F1-2 and MCS-6-27 antibodies were immobilized on a nitrocellulose strip each separated by ~4 mm. To demonstrate the specificity of the antibodies employed as capture and detector reagents, three different sets of conjugation pads were prepared containing F1-51 alone, BoB-92-32 alone and F1-51 and BoB-92-32 together at a ratio of 1:2. Each set of conjugation pads were tested with BoNT/A alone, BoNT/B alone, BoNT/A and /B together or with phosphate buffer (PB) alone. All toxins were used at a concentration of 100 ng/mL. As shown in Fig. 3, all capture and detector antibodies demonstrated a high level of specificity. BoNT/A was detected only when gold-conjugated F1-51 was present on the conjugation pad (Fig. 3A and C). When BoNT/A was applied to a conjugation pad containing only BoB-92-32, the toxin was not detected (Fig. 3B), demonstrating that BoNT/A toxin is not recognized by the BoNT/B-specific detector antibody. These results are consistent with those observed in the single serotype LFDs described above. The same held true for the detection of BoNT/B. BoNT/B was detected only when BoB-92-32 was present on the conjugation pad (Fig. 3B and C) and was not recognized by the BoNT/A detector antibody (Fig. 3A). These results demonstrate that the antibodies used as capture and detector antibodies for this LFD can distinguish between BoNT/A and /B serotypes and are consistent with earlier ELISA observations (Scotcher et al., 2010; Stanker et al., 2008; Yokota, 2010).

Finally, a titration from 100 ng/mL of each toxin to 0.2 ng/mL revealed that with the dual detector strip, a limit of detection for both BoNT/A and /B was reached at approximately 10 ng/mL of toxin (data not shown). These results are the first to demonstrate sensitive and selective detection of both BoNT/A and /B using a single lateral flow device.

3.3. Detection of BoNT in milk, apple and orange juice

To determine the utility of the dual BoNT/A/B LFD in real-world samples, we spiked whole, 2%, and 1% milk with BoNT/A and /B (500 ng/mL of each toxin) then serially diluted them with the appropriate milk to 100, 50, 25, 10 and 5 ng/mL. Prior to testing, spiked milk samples were further diluted 10-fold with double deionized water. With these diluted samples, the LFD showed the greatest sensitivity with 1% milk samples achieving a limit of detection of 100 ng/mL (concentration before dilution) for both BoNT/A and B (Table 1). In contrast, BoNT/A could be detected in 2% and whole milk at a spike level of 100 and 500 ng/mL, respectively. BoNT/B could be detected at a spike level of 500 ng/mL in 2% milk, but could not be detected in whole milk.

Given that the LFD demonstrated the greatest sensitivity with the least fatty milk samples, we attempted to further reduce the fat content of the milk samples by centrifugation. Whole, 2% and 1% milk samples were spiked at 1 μg/mL of each toxin, and then centrifuged at 4 °C. The resulting fatty layer was aspirated and serial dilutions of the spiked samples were prepared in defatted milk and applied directly to the LFD. As expected, removal of the fatty content greatly improved the sensitivity of the LFD. BoNT/A was detected at 10 ng/mL in defatted whole and defatted 1% milk and at a limit of 5 ng/mL in defatted 2% milk (Table 1). BoNT/B was detected at 25 ng/mL in defatted whole milk and at 10 ng/mL in both 2% and 1% defatted milk (Table 1). It should be noted that these defatted samples flowed faster and more evenly than the diluted milk samples. Overall, for the milk samples, fat removal *versus* sample dilution resulted in greater sensitivity.

BoNT/A/B spiked (500 ng/mL) apple (Fig. 4A–B) and orange juices (Fig. 4C–D) were also evaluated with our LFD. Following the spike with BoNT/A and B, both juices were brought to a neutral pH using 1 M NaOH, then serially diluted from 1 μg/mL to 10 ng/mL in neutralized juice. Apple juice was directly tested, and a limit of detection of 25 ng/mL and 10 ng/mL for BoNT/A and /B, respectively was achieved. Dilution of the spiked apple juice with a phosphate buffer did not improve assay performance for either BoNT/A or /B. The lowest level of detection following dilution was from samples with an initial spike of 50 ng/mL for BoNT/A and 10 ng/mL for BoNT/B.

Orange juice samples were diluted 2-fold with a phosphate buffer prior to testing. Both BoNT/A and B could be detected

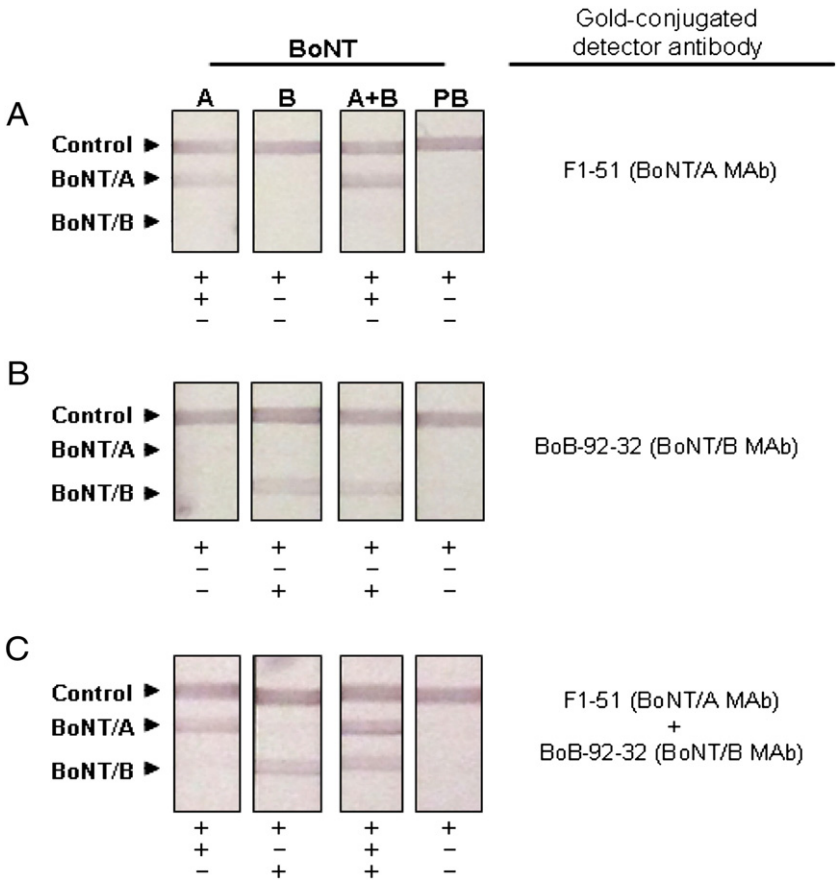

Fig. 3. A single device for detection of both BoNT/A and B. Lateral flow strips each with control, anti-BoNT/A and anti-BoNT/B capture lines were prepared with gold conjugated BoNT/A specific mAb F1-51 (panel A), gold conjugated BoNT/B specific mAb BoB-92-32 (panel B) or both gold conjugated BoNT/A and B mAbs (panel C). Samples containing BoNT serotype-A alone (Lane A), BoNT serotype-B alone (Lane B), a mixture of BoNT Serotype-A and -B (Lane A + B) or sample phosphate buffer alone (Lane PB) were applied. + indicates resolution of gold-conjugated antibody by immobilized control or test antibody; − indicates no resolution of gold-conjugated antibody by immobilized control or test antibody.

in samples spiked at 25 ng/mL before dilution, but not lower. The limit of detection following centrifugation remained at 25 ng/mL for both BoNT/A and B. Thus removal of particulate pulp in orange juice did not improve the sensitivity of the assay for either toxin.

Table 1
Limit of detection of BoNT/A and B in spiked beverages using a LFD.

	BoNT/A (ng/mL)	BoNT/B (ng/mL)
Whole milk	500[a]	Not detected
Whole milk, defatted	10	25
2% milk	100[a]	500[a]
2% milk, defatted	5	10
1% milk	100[a]	100[a]
1% milk, defatted	10	10
Apple juice	25	10
Apple juice, diluted	50[b]	10[b]
Orange juice, diluted	25[b]	25[b]
Orange juice, supernatant	25	25

[a] Spike level represents the amount of BoNT/A and B before a 10-fold dilution of the sample.
[b] Spike level represents the amount of BoNT/A and B before a two-fold dilution of the sample.

4. Discussion

Naturally occurring *C. botulinum* infection in the United States is a rare, but a serious condition. Foodborne botulism occurs sporadically throughout the country and is most often related to home-canned food, where the bacteria proliferate within the anaerobic environment (Sobel, 2005). A recent epidemiological study of wound botulism noted a 20-fold rise in known cases over a 10 year period, mostly attributed to injection drug users (Werner et al., 2000). Finally, attempts by bioterrorists to weaponize BoNT have been well documented in many countries (Arnon et al., 2001; Swaminathan, 2011). The most recent occurrence was in Japan, when, over a five year period, three attempts were made to disseminate aerosolized toxin in downtown Tokyo and at a U.S. military base in Japan (Arnon et al., 2001).

Given the public health threat of BoNTs many international groups have sought to develop alternative diagnostic assays to offset the labor-intensive mouse bioassay. The majority of these efforts focus on improving the sensitivity and selectivity of antibody based immunoassays. The use of BoNT serotype specific antibodies as part of diagnostic immunoassays has proven capable of resolving specific BoNT

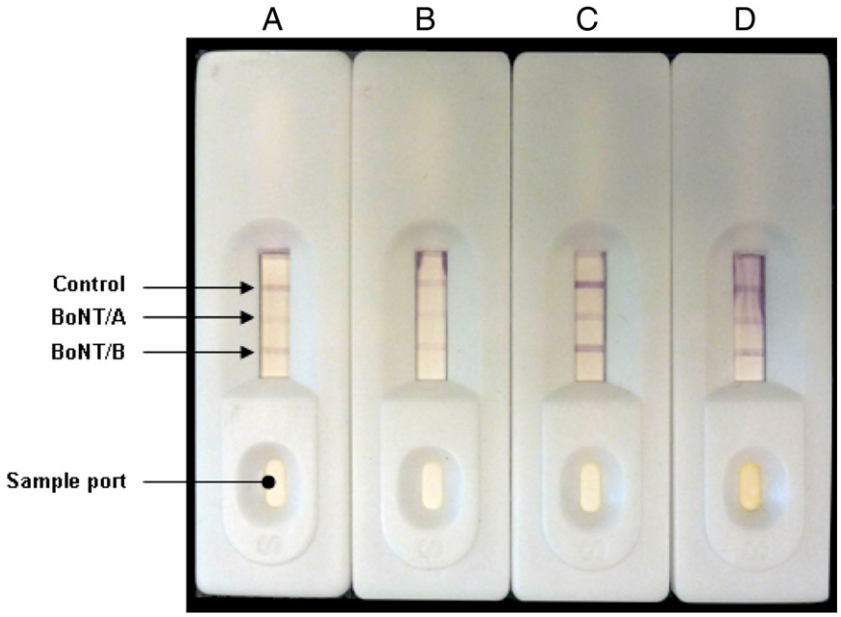

Fig. 4. Representative LFD for detection of BoNT Serotype-A and -B in spiked beverages. Undiluted apple juice (A), diluted apple juice (B), centrifuged orange juice (C) and diluted orange juice (D) all with an initial spike level of 500 ng/mL of BoNT/A and /B are shown. Fifty microliters of diluted sample was added at the sample port.

serotypes present at pg/mL in various matrices (Szilagyi et al., 2000; Sharma et al., 2006; Brunt et al., 2010; Brooks et al., 2011). Still others have exploited the endopeptidase activity of the toxin for detection using *in vitro* assays (Wictome et al., 1999; Rasooly and Do, 2008). While many of these assays approach the sensitivity of the mouse bioassay they still require specialized equipment and trained personnel. The development of highly sensitive BoNT detection assays as part of an overall bio-defense strategy should also include inexpensive portable diagnostic devices with simple visual verification for use by minimally trained personnel. A rapid colorimetric BoNT LFD would be of value to both emergency first responders in the assessment of possible contamination and to food processing facilities as part of routine quality assurance. A simple inexpensive BoNT LFD offers the potential to meet the need for rapid BoNT detection from a variety of substrates and settings.

Here we report the design and use of a single lateral flow device capable of detecting and distinguishing between BoNT/A and /B. The LFD demonstrated the greatest sensitivity for BoNT/A, detecting as little as 5 ng/mL in 2%, defatted milk. BoNT/B could be detected down to 10 ng/mL in spiked 1% and 2% defatted milk and undiluted apple juice. In contrast to currently available commercial LFDs, which utilize polyclonal antibodies that are cross reactive for BoNT/A and /B, our device can distinguish between BoNT/A and /B serotypes as it uses two sets of highly specific monoclonal antibody pairs. Recently, Sharma et al. evaluated the Alexeter Technologies BoNT/A/B strip, which cannot distinguish between the two serotypes (Sharma et al., 2005). In these studies, the Alexeter strip demonstrated a lower limit of detection of 100 ng/mL when spiked milk products were diluted and 10 ng/mL when they were defatted. The device developed here achieved similar sensitivities in milk, but outperformed the Alexeter Technologies strip in spiked orange juice samples by four-fold, detecting both BoNT/A and /B in orange juice spiked at 25 ng/mL. Gessler et al. evaluated the BioThreat Alert BoNT/A/B test strip, available from Tetracore, with a number of spiked clinical samples (Gessler et al., 2007). Interestingly, the test could not detect purified toxin, suggesting that the antibodies used in the strip are likely specific for epitopes of the BoNT complex and not the actual toxin itself. Both capture antibodies used in our device, F1-2 and MCS-6-27, recognize specific epitopes on the heavy chains of BoNT/A and B, respectively (Scotcher et al., 2009, 2010), and are thus capable of detecting purified toxin as well as crude toxin preparations.

Colloidal gold labeling of antibodies is one of the most widely employed strategies for building lateral flow devices because it is relatively inexpensive and very stable in its dried form. While we employed gold-labeled antibodies in the system described here, colored latex beads could alternatively be incorporated and would provide a color-coded result that would further simplify interpretation of the results. Studies are in progress to add other serotype specific antibodies, such as BoNT/E and /F, that will add further value to our detection device.

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