Evaluation of Lateral-Flow Clostridium botulinum Neurotoxin Detection Kits for Food Analysis

Shashi K. Sharma,1* Brian S. Eblen,1 Robert L. Bull,2 Donald H. Burr,3 and Richard C. Whiting1

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, Maryland 207401; Biological Defense Research Directorate, Naval Medical Research Center, Silver Spring, Maryland 209102; and National Center for Food Safety and Technology, Summit-Argo, Illinois 605013

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Clostridium botulinum produces seven (A to G) structurally related but antigenically distinct protein neurotoxins. These botulinum neurotoxins (BoNTs) are the causative agents of botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29). There has been much effort by the food industry to ensure that food treatment processes prevent botulism (3, 18, 21, 22, 29).

The suitability and sensitivity of two in vitro lateral-flow assays for detecting Clostridium botulinum neurotoxins (BoNTs) in an assortment of foods were evaluated. Toxin extraction and preparation methods for various liquid, solid, and high-fat-content foods were developed. The lateral-flow assays, one developed by the Naval Medical Research Center (Silver Spring, MD) and the other by Alexeter Technologies (Gaithersburg, MD), are based on the immunodetection of BoNT types A, B, and E. The assays were found to be rapid and easy to perform with minimum requirements for laboratory equipment or skills. They can readily detect 10 ng/ml of BoNT types A and B and 20 ng/ml of BoNT type E. Compared to other in vitro detection methods, these assays are less sensitive, and the assessment of a result is strictly qualitative. However, the assay was found to be simple to use and to require minimal training. The assays successfully detected BoNT types A, B, and E in a large variety of foods, suggesting their potential usefulness as a preliminary screening system for triaging food samples with elevated BoNT levels in the event of a C. botulinum contamination event.
detect analytes in the nanogram range, they are user friendly, relatively inexpensive, and ideally suited for on-site testing by minimally trained personnel and can be adaptable for high-throughput laboratory or field use. However, lateral-flow assays do have a major limitation: they deliver only qualitative results, i.e., a “yes” or “no” answer detected by the human eye. Manufacturers have been developing rapid tests that deliver quantitative results, some of which are being commercialized with some success. The most common readers translate line intensity into analyte concentration and use either colorimetric reflectance or a charge-coupled-device camera to measure the signal intensity. However, like other rapid assays, sophisticated instruments limit the detection time and require trained analysts. Other commercially available readers use the emittance of a fluorescent label that is fixed to conjugate particles. In this study, we evaluated two immunodetection kits for the detection of BoNT type A (BoNT/A), BoNT/B, and BoNT/E in foods and developed procedures to extract the toxin from food samples.

### MATERIALS AND METHODS

#### Culture conditions and growth of C. botulinum strains.

Type A strains were grown anaerobically in a Bacto cooked meat medium (CMM; Difco Laboratories, Detroit, MI) overnight at 35°C. One milliliter of overnight-grown culture was transferred to Trypticase-peptone-glucose-yeast extract broth (TPGY) and grown at 35°C for 5 days. The culture material was clarified by centrifugation through a 0.45-µm-pore-size bacteriological Acrodisc filter. Culture filtrate was stored at 4°C until used.

#### Sample preparation.

Control samples of botulinum neurotoxin complexes A, B, and E (range, 0.2 to 1 µg/ml) were prepared in 500 µl of sample buffer supplied with each test kit. For liquid food samples such as orange juice, bottled water, soft drinks, vanilla extract, and apple juice, a 5-ml food sample was spiked with 100 ng/ml of pure botulinum neurotoxin complex A, B, or E and incubated for 30 min at room temperature (25°C). The samples were then diluted to a ratio of 1:5 in a sample buffer (0.01 M phosphate buffer, pH 7.4). After incubation, the samples were centrifuged at 7,000 × g for 30 min at 4°C to remove solid particles and/or the lipid layer. Subsequently, 500 µl of the supernatant was thoroughly mixed with 500 µl of sample buffer in a glass test tube and used for the assay.

High-fat-content and viscous foods such as ice cream, milk, and honey were spiked with 100 ng/ml of pure botulinum neurotoxin complex A, B, or E and incubated for 30 min at room temperature (25°C). The samples were then diluted to a ratio of 1:5 in a sample buffer (0.01 M phosphate buffer, pH 7.4). After incubation, the samples were centrifuged at 7,000 × g for 30 min at 4°C to remove solid particles and/or the lipid layer. Subsequently, 500 µl of the supernatant was thoroughly mixed with 500 µl of sample buffer in a glass test tube and used for the assay.

### RESULTS

#### Sensitivity.

A series of control samples containing BoNT/A, -/B, and -/E (0.2 to 100 ng/ml) was tested to determine the sensitivity of the test. The sensitivity tests for BoNT/A and -/B and those for BoNT/E were performed separately. No colored lines were observed in the sample windows for samples containing 0.2 to 9 ng/ml of BoNT/A or -/B or for samples con-
taining 0.2 to 19 ng/ml of BoNT/E. Positive red lines appeared in the sample windows for the samples containing 10 ng/ml of either BoNT/A or -/B. In the case of BoNT/E, the red line appeared with samples containing 20 ng/ml of the toxin, suggesting that the test can detect concentrations of BoNT/A and -/B as low as 10 ng/ml and of BoNT/E as low as 20 ng/ml.

Milk products. The Alexeter Technologies kit showed poor filtration and migration of the high-fat-content samples compared to that of the NMRC kit. These samples filtered poorly through the filtration device and were unable to migrate to the control window (Table 1). However, a clear red-colored line appeared in the sample window, indicating a positive reaction. With the NMRC kit, the samples, except for whipping cream, reached the sample and control windows within 15 min (Table 2). Both BoNT/A and -/B assay kits exhibited similar results. In the case of the BoNT/E assay, samples such as whipping cream, half-and-half, and raw milk showed negative results. Very slow migrations of the samples were observed (Table 2).

To improve the filtration of the products such as pasteurized milk, 1% milk, half-and-half, and whipping cream, the samples were centrifuged at 7,000 × g for 10 min. The supernatant was carefully taken out through a pipette and directly applied on the kit. In another experiment, samples were diluted to a 1:10 ratio in a sample buffer containing 0.01 M phosphate-buffered saline, pH 7.4 (final concentrations, 10 ng/ml for BoNT/A and -/B and 20 ng/ml for BoNT/E). Both centrifugation and further dilution markedly improved the filtration process, and all four samples were able to filter through the device and migrate (Tables 1 and 2).

It was assumed that the slow filtration could be due to the

![Schematic representation of the lateral-flow assay.](image)

**FIG. 1.** (A) Schematic representation of the lateral-flow assay. A mixture of target BoNT and antibody migrates by capillary force to the membrane toward the capture zone. (B) Overview of a lateral-flow assay. The antibodies specific to a toxin immobilized onto a membrane at the test line position as shown in the sample window in the panel. These antibodies will react only with the BoNT toxin. There is also a control line, as shown in the control window in the figure, which indicates whether the test has worked or not. If a sample is positive for BoNT, there will be two lines in the viewing windows (sample and control); if negative, there will be only one at the control line position. If there is just one line at the test line position, the result should be ignored and considered invalid.

<table>
<thead>
<tr>
<th>Table 1. Detection of BoNT/A and -/B in milk samples using Alexeter Technologies-developed lateral-flow in vitro bioassay*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk product</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1% Fat*</td>
</tr>
<tr>
<td>Fat free</td>
</tr>
<tr>
<td>Whipping cream</td>
</tr>
<tr>
<td>Milk*</td>
</tr>
<tr>
<td>Half-and-half</td>
</tr>
<tr>
<td>2% Fat</td>
</tr>
<tr>
<td>Raw milk</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

*The food samples were spiked with 10 ng/ml of BoNT/A or BoNT/B. The results were recorded after 15 and 30 min. The results represent the mean values of three samples for each food type (n = 3). No mixed results were observed.

*An asterisk indicates that the sample reached to the test window but not to the control window.
presence of lipid in the samples and that an organic solvent may dissolve or separate the fat from the aqueous solution. Therefore, we used the organic solvent methanol to partition the toxin. After extraction, the lower aqueous layer was applied to the kit. No color line appeared in either the sample or the control window. Similar results were observed for both manufacturers’ kits (Tables 1 and 2).

**Solid foods, liquid foods, and seafood.** A wide variety of food samples, including liquid, solid, and semisolid food and seafood, were tested. Of the 33 solid and liquid foods, only 10 foods showed a positive test endpoint within 15 min when used in undiluted forms. Ice cream, orange juice, and honey gave negative results with the Alexter Technologies kit. These food samples were found positive after being diluted to a ratio of 1:10 in a sample buffer (Table 3).

With the NMRC assay kit, a majority of liquid foods such as orange juice, bottled water, Coca-Cola, and vanilla extract showed positive results within 15 min. Solid foods such as broccoli, allspice, cinnamon, catfish nugget, snow crab, and Atlantic salmon also showed positive reactions within 15 min.

**Detection of BoNT/A, -/B, and -/E in milk samples using NMRC-developed lateral-flow in vitro bioassay.**

<table>
<thead>
<tr>
<th>Milk product</th>
<th>BoNT/A results for samples that were:</th>
<th>BoNT/B results for samples that were:</th>
<th>BoNT/E results for samples that were:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted</td>
<td>Diluted</td>
<td>Centrifuged</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
<td>15 min</td>
</tr>
<tr>
<td>1% Fat</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fat free</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Whipping cream</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Milk</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Half-and-half</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>2% Fat</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Raw milk</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Control</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

*Food samples were spiked with 10 ng/ml of BoNT/A or BoNT/B and 20 ng/ml of BoNT/E. The results were recorded after 15 and 30 min. The results represent the mean values of three samples for each food type (n = 3). No mixed results were observed.

The food samples of honey and infant formula were unable to reach the sample windows and showed negative reactions in the first 15 min of the test. In the case of apple juice, although the sample was filtered through a nitrocellulose membrane, the membrane turned slightly red, making it difficult to read the result. Only those viscous samples which were found negative within 30 min in undiluted form in a sample buffer were diluted (final concentrations, 10 ng/ml for BoNT/A and -/B and 20 ng/ml for BoNT/E). All diluted food samples turned positive within 15 or 30 min of test time (Table 4).

Unlike the BoNT/A and -/B kits, in which the times to positive observation vary, the results virtually remained unchanged even after 30 min for BoNT/E. The undiluted liquid sample either did not filter or was unable to reach the control window, leaving a negative result. However, upon dilution, in a manner similar to that seen in the assays for BoNT/A and -/B, food samples were able to reach the control windows. For BoNT/E, although the test was considered positive and both sample and control window lines were clear and visible, the color was less intense than that for BoNT/A and BoNT/B (Table 4).

**Detection of cultured toxin and specificity.** Seventy-seven strains of *Clostridium botulinum* type A isolated from different environmental and clinical samples were chosen for the detection of the cultured toxin. These strains are isolates of outbreaks and are from a Food and Drug Administration *Clostridium botulinum* depository. The culture filtrate from each isolate was diluted 100-fold in a 0.01 M phosphate buffer, pH 7.4, before being used in the assay. Further attempts to dilute the cultured toxin (1 × 10^3 dilution) failed, and negative results were observed. Except for strain CS-A, all strains showed positive results when grown in TPGY medium. Culture filtrates of four strains, i.e., SKOR1A (8833B1), SKOR@A (8933E1), CS-A, and 8-A, grown in a CMM medium were found negative for the presence of BoNT/A. In order to test for false-positive results, we used denatured BoNT/A complex (heated at 100°C for 10 min), pure BoNT/E and -/F complexes, tap water, bovine serum albumin, purified hemagglutinin-33 (from BoNT/A complex), and culture filtrates of 26 other non-botulinum strains, including strains of *Clostridium acetobutylicum*, *Clostridium chauvoei*, *Clostridium sporogenes*, *Clostridium*
histolyticum, Clostridium tetani, Clostridium bifermentans, Clostridium sordellii, Clostridium perfringens, Bacillus subtilis, Bacillus cereus, and Bacillus megaterium as interfering agents. No false positives were observed for 26 of the culture filtrates of 26 strains or for other interfering agents except for BoNT/F. A very faint positive line appeared in sample windows with BoNT/F samples.

**DISCUSSION**

Immunoassay technologies are ideal for the qualitative and quantitative detection of many types of proteins and pathogens in complex matrices (1, 6). Lateral-flow assays have been used extensively as diagnostic tools for monitoring toxins (7, 32, 33). However, their effectiveness is confirmed only for toxins in serum and environmental samples. We wanted to evaluate lateral-flow assays as an early warning tool for the detection of C. botulinum toxins in a variety of foods. In this investigation, two assay kits appear to be similar in technology but performed slightly differently in our experiments. The major difference observed seems to be in the mechanical assemblies of the filtration devices and the nitrocellulose membrane supports. For BoNT/A, the assay sensitivities were found to be similar for both the Alexeter Technologies assay kit and the NMRC assay kit. During the cross-reaction studies, we observed a positive reaction in the BoNT/A kit with BoNT/B neurotoxin. When tested for sensitivity, the BoNT/A kit was found to be equally sensitive for BoNT/B neurotoxin. The cross-reaction between the BoNT/A and BoNT/B antibodies has been reported in the literature (8, 10). Alexeter Technologies, Inc. markets kits only for BoNT/A. The sensitivity limit of the NMRC BoNT/E kit was found to be 20 ng/ml. The assay’s sensitivities for BoNT/A, -B, and -E were much less than those of the mouse bioassay and therefore, for the comparison, we did not perform the mouse bioassay.

The assays were visualized as red lines created by the bound colloidal gold; therefore, sensitivity is limited to what can be seen by the human eye and is lower than that of sensitive instrumentation such as a spectrophotometer or a fluorescent reader. Typically, an arbitrary quantitation of the detection
The lethal dose of botulinum toxin for humans is not precisely known. The estimated lethal amount of crystalline type A toxin for a 70-kg human would be approximately 0.09 to 0.15 μg intravenously or intramuscularly, 0.70 to 0.90 μg inhalationally, or 70 μg orally (4). The detection limit of the mouse bioassay is 10 pg/ml of pure BoNT/A (11). We found that the BoNT/A and -/B assay kits can readily detect 10 ng/ml, while the BoNT/E kit can detect as low as 20 ng/ml of BoNT/E. Although these assays exhibited sensitivities less than the detection limit of the mouse bioassay, they are capable of detecting the toxin concentration that can cause botulism disease symptoms in humans. Therefore, they can be considered for large-scale or presumptive test screening to be followed by a confirmatory mouse bioassay.

Certain milk-based samples, such as whipping cream, half-and-half, and raw milk, when tested in undiluted form, exhibited low filtration or sometimes virtually no filtration with these test devices. Such problems were particularly encountered by the Alexeter Technologies kits. The test kits from NMRC performed relatively well in terms of filtration and sample migration of the analytes. They developed filtration and sample migration problems only with whipping cream. This was perhaps due to the high fat contents of these milk products (Table 5). In general, the filtration system prevents the solid particles and passes analytes (toxin) freely to the membrane. Lateral-flow assays work on the basis of a capillary process (2, 25) and, because of the action of capillary forces, the toxin has to migrate up the membrane to where ligands are immobilized in the capture zones. It seems that the high fat contents of the samples prevent the capillary flow of the samples. However, several other factors could cause negative results, including the inhibition of antigen antibody reaction due the presence of inhibitory enzymes present in the samples.

The manufacturers’ recommended time for recording the result is 15 min. We considered an extra 15 min of incubation, because undiluted high-fat-content foods were unable to reach the control window within 15 min. In almost all cases, an extra 15 min was sufficient for the toxins to reach the control windows even with slow migration of the samples. Alternatively, dilution or centrifugation may also speed up the migration process observed in certain milk-based samples (Table 4). Both sample and control lines appeared, indicating a positive reaction. Therefore, we recommend that at least a 30-min test time be used for high-fat-content food samples. The Alexeter Technologies kit instructions indicate that colored lines that appear after 20 min are not valid and should be ignored. We also observed slow filtration for some semisolid and solid food samples similar to that of the milk-based samples. Since these foods do not contain amounts of fat sufficient to restrict the flow of the samples through membrane and their flows were virtually similar to those of milk-based samples, it is apparent that fat is not the only factor that is responsible for slow filtration.

These lateral-flow tests can detect up to 100-fold dilutions of the cultured toxin in both TPGY medium and CMM, indicating that undiluted cultured toxins of foods can be tested for a qualitative identification of BoNT. The choice of medium and its constituents can also affect the overall results of the assay. For example, of the 77 strains grown in CMM, the assay showed three false-negative results. In the TPGY medium, no false negatives were observed, and all strains (except CS-A) showed positive reactions. Except for BoNT/F, other potentially interfering agents did not give false-positive results, perhaps indicating that a weak cross-reaction of BoNT/F antibodies occurs with BoNT/A and -/B.

The ELISA method, which can detect from 0.1 to 1 ng/ml (10 to 100 MLD) of the toxin in a variety of samples, including food, has been widely used by researchers (15) and is commercially available from Metabiologics, Inc. The modified ELISA was compared with the AOAC International method and proved useful for screening a large quantity of samples. The assay takes approximately 5 h to complete. The major disadvantage of ELISA is that it is relatively time and labor intensive. Unlike the typical ELISA, in which either antibodies or antigens are directly bound to a polystyrene or polyvinyl microplate, electrochemiluminescence detection offers relatively better sensitivity (0.05 to 1.0 ng/ml, ~10 MLD) through a process in which antibodies are attached on the magnetic particles in a large volume of sample suspension and then captured and bound in a small area. Electrochemiluminescence detection provides increased sensitivity due to high luminescence-signal-to-noise ratios. However, the assay performance could vary significantly depending on the sample matrices. Because of this, matrix-specific positive and negative control samples are used to establish standard curves and cutoff values. An expensive instrumentation is also a major limitation with

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**TABLE 5. Milk product contents**

<table>
<thead>
<tr>
<th>Milk product</th>
<th>Serving size (ml)</th>
<th>Total fat (g)</th>
<th>Saturated fats (g)</th>
<th>Cholesterol (mg)</th>
<th>Sodium (mg)</th>
<th>Carbohydrates (g)</th>
<th>Dietary fiber (g)</th>
<th>Sugars (g)</th>
<th>Proteins (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Low fat</td>
<td>50</td>
<td>0.52</td>
<td>0.31</td>
<td>2.08</td>
<td>27.08</td>
<td>2.50</td>
<td>0.00</td>
<td>2.29</td>
<td>1.67</td>
</tr>
<tr>
<td>Fat free</td>
<td>50</td>
<td>0.00</td>
<td>0.00</td>
<td>1.04</td>
<td>27.08</td>
<td>2.50</td>
<td>0.00</td>
<td>2.29</td>
<td>1.67</td>
</tr>
<tr>
<td>Whipping cream</td>
<td>50</td>
<td>16.67</td>
<td>11.67</td>
<td>66.67</td>
<td>16.67</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>50</td>
<td>1.67</td>
<td>1.04</td>
<td>7.29</td>
<td>26.04</td>
<td>2.50</td>
<td>0.00</td>
<td>2.29</td>
<td>1.67</td>
</tr>
<tr>
<td>Half and half</td>
<td>50</td>
<td>5.00</td>
<td>3.33</td>
<td>25.00</td>
<td>25.00</td>
<td>3.33</td>
<td>0.00</td>
<td>1.67</td>
<td>1.67</td>
</tr>
<tr>
<td>2% Reduced fat</td>
<td>50</td>
<td>1.04</td>
<td>0.63</td>
<td>4.17</td>
<td>26.04</td>
<td>2.50</td>
<td>0.00</td>
<td>2.29</td>
<td>1.67</td>
</tr>
<tr>
<td>Raw milk</td>
<td>50</td>
<td>1.85</td>
<td>0.935</td>
<td>5.00</td>
<td>25.00</td>
<td>2.20</td>
<td>0.00</td>
<td>2.63</td>
<td>1.75</td>
</tr>
</tbody>
</table>

*Values are normalized to 50-ml serving sizes for uniform representation.

*Product descriptions are based on the labels attached to the products.*

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**sensitivities of these assays is done by assigning “plus” and “minus” values, with the increasing intensity of the red line assigned a plus value. Besides the somewhat qualitative nature of this process, the weak-positive-result values can vary based on the skill of the technician responsible for validating a given lot of assays.**
the assay. Lateral-flow assays are simple to use and require minimal training. They are relatively cost effective and can be used as first-responder detection kits. Although these devices sound ideal, they have limitations. Key limitations are that they are less sensitive than other in vitro detection systems and that the assessments of results are strictly qualitative. New-generation lateral-flow assays based on fluorescence have substantially greater sensitivities and dynamic ranges than did the ones used in this study. Nevertheless, the development of such assays has been limited by the need for stable dyes that do not cause sample interference and by the fact that the instrumentation required was both complex and expensive.

In summary, the performance of the LFDs indicates that these assays are suitable as screening tools for a variety of foods potentially contaminated with BoNT. The speed of the assays are suitable as screening tools for a variety of foods potentially contaminated with BoNT. The speed of these assays is critical for rapid detection of insecticides and pesticides in food samples. Nevertheless, the development of such assays has been limited by the need for stable dyes that do not cause sample interference and by the fact that the instrumentation required was both complex and expensive.

REFERENCES