Use of a new functional dual coating (FDC) assay to measure low toxin levels in serum and food samples following an outbreak of human botulism

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Clostridium botulinum type A toxin is the most prevalent cause of naturally occurring outbreaks of human botulism in the world. The active dichain neurotoxin molecule is composed of a heavy chain (H-chain) of ~100 kDa with the carboxy-terminal end consisting of a receptor-binding (H₉) domain, while the amino-terminal (H₈) domain is linked by a critical disulfide bond to a light chain (L-chain) of ~50 kDa. Although the mouse bioassay (MBA) is traditionally used to confirm the presence of toxin in serum or food, its sensitivity is insufficient to detect low toxin levels in approximately 30 to 60 % of botulism patients. A novel FDC (functional dual coating) microtitre plate immuno-biochemical assay, which quantifies botulinum toxicity by measuring the H₉ domain linked with L-chain endopeptidase activity, was modified to allow human serum (lysed or unlysed) to be tested without interference from the matrix, with toxin detection down to 0.03 mouse LD₅₀ per ml serum or 0.13 pg ml⁻¹ using just 100 µl of clinical samples. The assay was specific for type A toxin and could additionally be applied to whole blood and food samples. Low levels of 1 to 2 mouse LD₅₀ per ml serum of type A toxin were quantified for the first time using the modified FDC assay in two severely intoxicated UK patients who required mechanical ventilation and antitoxin. Toxin levels in recovered food sample extracts were also detected and one MBA-negative sample was found to contain 0.32 LD₅₀ per ml extract. The FDC assay provides a real alternative for public health laboratories to unambiguously confirm all cases of type A botulism and, due to its sensitivity, a promising new tool in toxin pharmacokinetic studies.

INTRODUCTION

Botulism remains a severe but rare life-threatening disease in the UK (McLauchlin et al., 2006). The most common presentations are wound, infant or food botulism. Occasionally adult intestinal toxæmia is seen as a result of ingesting spores, but this typically only affects individuals with an underlying gastrointestinal condition (Sheppard et al., 2012). During food-borne botulism, ingested toxin is absorbed across the gut to reach the blood circulation and then redistributed into the interstitial fluid, where it can diffuse into synaptic clefts of neuromuscular junctions and specifically bind to the nerve terminal via the toxin’s H₉ (receptor-binding) domain of the heavy (H) chain (Maksymowych & Simpson, 1998; Maksymowych et al., 1999; Simpson, 2004; Ahsan et al., 2005). Following uptake into the cell, the disulfide bond linking the H₈ domain of the H-chain to the light (L) chain is broken by the reducing conditions with subsequent activation and release of the L-chain endopeptidase within the cytosol. Specific cleavage of SNAP25₁₋₂₀₆ protein between amino acid residues Q₁₉₇ and R₁₉₈ (in the case of type A toxin) then inhibits neurotransmitter release, with a resultant nerve blockade (Schiavo et al., 2000; Simpson, 2004).

The clinical diagnosis of botulism is usually made on the early signs and symptoms, such as dry mouth, difficulty in swallowing, slurred speech, ptosis and ophthalmoplegia, which, if left untreated, progress to full-blown botulism with a symmetrical descending paralysis requiring mechanical ventilation. A trivalent (A, B and E) botulism antitoxin is given rapidly at the earliest indication to prevent any further progression and speed recovery (CDC, 1998; Browning et al., 2011; Vanella de Cuetos et al., 2011). The licensed antitoxin used in the UK is safely administered in the hospital setting and comprises purified despectated equine F(ab’)₂ fragments with high neutralizing potency (Novartis trivalent ‘Botulism Antitoxin Behring’ containing ≥187 500 IU anti-A, ≥125 000 IU...
anti-B and ≥12,500 IU anti-E, per vial). One or more serum samples are normally taken for analysis prior to antitoxin administration. Detection of toxins in these samples provides the unambiguous laboratory confirmation of a clinical diagnosis as well as identifying the bacterial species (McLauchlin et al., 2006), which helps trace the source of contamination. In November 2011 three cases of food botulism occurred in a family in the UK and, following the clinical diagnosis, trivalent antitoxin was given (Browning et al., 2011). A multidisciplinary investigation was required to conclusively identify the type A toxin in a jar of korma sauce as the vehicle of intoxication (Browning et al., 2011). An in vivo mouse bioassay (MBA) is traditionally performed in toxicological investigations. It involves injecting the sample (patient serum, other clinical samples or extracts from food) intraperitoneally with or without mono-specific antitoxins (in a maximum total injection volume of 0.5 ml) and observing for signs of botulism or death (CDC, 1998; Soloman & Lilly, 2001; Sanford et al., 2010; Cheng et al., 2012). The MBA takes up to 4 days (96 h) to complete and, although effective at measuring high levels of toxin (within 24 h), fails to confirm the clinical diagnosis of botulism in approximately 30 to 60% of cases when serum toxin levels fall below the assay’s detection limit (Woodruff et al., 1992; McLauchlin et al., 2006; Wheeler et al., 2009). The signs of neurotoxicity detected by the MBA may not unambiguously allow differentiation between botulism and autoimmune neuropathies, such as Guillain–Barré syndrome, unless specific neutralization using a botulinum antitoxin is seen (van den Berg et al., 1994; Susuki et al., 2001; Mazuet et al., 2012). The assay may also be unsuitable if appropriate unlysed serum samples are not available in adequate quantities or some toxin degradation has occurred during transit (ASM, 2003; McLauchlin et al., 2006; Rowlands et al., 2010). As a negative laboratory test result for specific neurotoxin detection in patient serum cannot refute the clinical diagnosis of botulism (Wheeler et al., 2009), the development of a more sensitive assay, which can overcome the inadequacies of the conventional test, would be highly beneficial, especially when analysing clinical samples containing low toxin levels.

A simple new method utilizing dual-coated microtitre plates (monoclonal antibody to the toxin’s Hc domain plus SNAP251–206 protein substrate) has recently been devised to specifically quantify biologically active toxin molecules in commercially available therapeutic botulinum type A toxin preparations (Liu et al., 2012). The FDC (functional dual coating) assay showed high sensitivity down to 40 fg ml⁻¹ [or 130 aM limit of detection (LoD)] and could replace the conventional mouse LD₅₀ test.

We describe here for the first time the development of this novel FDC assay with a range of clinical samples obtained from outbreaks of food-borne botulism (Browning et al., 2011; Vossen et al., 2012). The modified FDC test allowed for the sensitive, specific detection and quantification of botulinum type A toxin in serum, lysed serum and food samples in an animal-free assay.

**METHODS**

**Human serum samples.** A range of coded clinical serum samples were provided by the Laboratory of Gastrointestinal Pathogens, Health Protection Agency, Centre for Infections, London, UK. They had previously been subjected to conventional MBA testing (Browning et al., 2011; Vossen et al., 2012) and are listed in Table 1. All samples were taken prior to antitoxin administration and stored frozen. Freeze-dried human serum (06/144, NIBSC, UK) was reconstituted with 1 ml distilled water and, following centrifugation, used as a source of serum for assay development and as a toxin-spiked reference during testing. Whole blood haemolysate (95/528, NIBSC, UK) was reconstituted with 1 ml distilled water prior to use.

**Food sample extracts.** A range of food sample extracts were provided by the Laboratory of Gastrointestinal Pathogens, Health Protection Agency, Centre for Infections, London, UK. These food remants were originally obtained from rubbish bins and recycling boxes during a type A botulism outbreak in November 2011. Samples had previously been subjected to conventional MBA laboratory testing (Browning et al., 2011) and are listed in Table 2. Pita bread, houmous and a spat-out food remnant (chicken and korma sauce) were all tested.

**Table 1. Clinical serum samples for testing in the FDC assay**

<table>
<thead>
<tr>
<th>Confirmed botulism diagnosis</th>
<th>Sample details</th>
<th>MBA</th>
<th>Sample code/year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A botulism (1st case)*</td>
<td>Serum (taken on day of admission)</td>
<td>Not tested†</td>
<td>S4/2011</td>
</tr>
<tr>
<td>Type A botulism (1st case)*</td>
<td>Serum (taken 1 day after admission)</td>
<td>Not tested†</td>
<td>S5/2011</td>
</tr>
<tr>
<td>Type A botulism (2nd case)*</td>
<td>Serum (taken 1 day after admission)</td>
<td>Not tested†</td>
<td>S6/2011</td>
</tr>
<tr>
<td>Type B botulism (control)‡</td>
<td>Serum</td>
<td>Positive for type B toxin</td>
<td>S1/2011</td>
</tr>
<tr>
<td>Type B botulism (control)‡</td>
<td>Serum</td>
<td>Positive for type B toxin</td>
<td>S2/2011</td>
</tr>
<tr>
<td>Non-botulism (control)</td>
<td>Serum</td>
<td>Negative</td>
<td>S3/2011</td>
</tr>
</tbody>
</table>

*Confirmed cases from the 2011 UK food-borne botulism outbreak (Browning et al., 2011).

†Severely haemolysed samples.

‡Type A-negative samples (Vossen et al., 2012).
extracted in gelatin (2 mg ml$^{-1}$)/phosphate (28 mM Na$_2$HPO$_4$) buffer pH 6.4, filtered (0.2 µm) and subsequently stored frozen (CDC, 1998).

Botulinum type A toxin reference material. Frozen aliquots of purified botulinum type A toxin, complex-free (Hall strain), were prepared by diluting a concentrated stock toxin solution containing $2.3 \times 10^8$ mouse LD$_{50}$ mg$^{-1}$ purchased from Metabiologics, to 20 000 mouse LD$_{50}$ mg$^{-1}$ in gelatin (2 mg ml$^{-1}$)/phosphate (50 mM Na$_2$HPO$_4$) buffer pH 6.5 (GP-buffer). Concentrated toxin was only handled within a class I safety cabinet. Botulinum toxin types B–G (Metabiologics) were prepared as previously described (Jones et al., 2009). Types B and E were complex-free while C1, D, F and G toxins were in the complex form.

Modiﬁed FDC assay. The FDC microtitre plate method was essentially used as originally described by Liu et al. (2012), with slight modifiﬁcation for use with human serum and food samples. Polystyrene Nunc Maxisorp 96-well plates (VWR International) were coated with 50 µl per well of pre-mixed 3 µg ml$^{-1}$ SNAP25$^{197}$ substrate (Stratech Scientiﬁc or USBiological, Cat. no. S1014-70Q) plus 1 µg ml$^{-1}$ monoclonal capture antibody 3D12 (Razai et al., 2005) in 50 mM carbonate buffer (pH 9.6), incubated overnight at 4°C in sealed plates, decanted and blocked with 300 µl per well of 50 g l$^{-1}$ skimmed milk powder (10 g l$^{-1}$ skimmed milk powder in PBST) was added; plates were then sealed and incubated at 37°C for 1 h (without stacking). After washing away any unbound material, 50 µl per well of reaction buffer (50 mM HEPES, pH 7.0; 0.5 %, v/v, Tween-20; 40 mM DTT) was added, then plates were individually sealed and incubated at room temperature for 20 h. The plates were then washed three times with PBST. Samples of serum, food or other extract were then diluted with an equal volume of sample buffer comprising 12.5 × PIC (protease inhibitor cocktail tablets, complete, EDTA-free, Roche Diagnostics, Cat. no. 04693132001) dissolved in 2.5% (w/v) skimmed milk powder in capture phosphate buffer (CP-buffer: 3.35 mM KH$_2$PO$_4$, 16.21 mM Na$_2$HPO$_4$, 136.89 mM NaCl; 0.05 %, v/v, Tween-20) for 1 h at 37°C. Plates were then washed three times with PBST. Samples of serum, food or other extract were then diluted with an equal volume of sample buffer comprising 12.5 × PIC (protease inhibitor cocktail tablets, complete, EDTA-free, Roche Diagnostics, Cat. no. 04693132001) dissolved in 2.5% (w/v) skimmed milk powder in capture phosphate buffer (CP-buffer: 3.35 mM KH$_2$PO$_4$, 16.21 mM Na$_2$HPO$_4$, 136.89 mM NaCl; 0.05 %, v/v, Tween-20), mixed and added to the first wells of the plate then serially diluted into 100 µl volumes of 6.25 × PIC dissolved in 1.25% (w/v) skimmed milk powder in CP-buffer. Plates were then sealed and incubated at 37°C for 90 min (without stacking). After washing away any unbound material, 50 µl per well of reaction buffer (50 mM HEPES; 20 µM ZnCl$_2$, pH 7.0; 0.5 %, v/v, Tween-20; 40 mM DTT) was added, then plates were individually sealed and incubated at room temperature for 20 h. The plates were then washed three times with PBST, blot dried, and 100 µl per well of neoepitope-specific detecting antibody (rabbit anti-SNAP25$^{197}$ at a 1 in 4000 dilution or alternatively 0.1 µg ml$^{-1}$ of affi nity-purified antibody) in antibody buffer (10 g l$^{-1}$ skimmed milk powder in PBST) was added; plates were then sealed and incubated at 37°C for 90 min without stacking. The plates were washed again, 100 µl per well of goat anti-rabbit-HRP (Perbio Science UK, Code no. 31460) at 1 in 16 000 dilution in antibody buffer from 50 % glycerol stock stored at −20°C was added, and the plates incubated as before. After washing, 100 µl per well substrate solution (50 mM citric acid pH 4.0, 0.91 mM ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diamin omonium salt], containing 0.015 %, w/v, hydrogen peroxide) was added and the colour allowed to develop at room temperature for 30 min. Following development, plates were briefly shaken and absorbance read at 405 nm using a suitable plate reader.

RESULTS

Optimization of the new FDC assay for use with clinical samples

Initial experiments, in which toxin-spiked serum samples were diluted in CP-buffer alone, identifi ed a considerable serum-induced interference. This inhibitory effect was maximal (100 % inhibition of toxin signal) at 1 in 8 sample dilution and could be overcome by adding skimmed milk powder (1.25 %, w/v, final concentration) to CP-buffer. An excess of protease inhibitor cocktail (6.25 × final concentration in CP-buffer plus skimmed milk) was also included to provide added protection against sample proteolytic enzyme activity during the initial 90 min capture step of the FDC assay.

To assess any interfering effect on assay detection deriving from the sample matrix, a control human serum sample, kept undiluted and spiked with a range of different toxin concentrations, was assayed in parallel to the same serum sample spiked with a single toxin concentration (4 LD$_{50}$ ml$^{-1}$) and serially diluted directly on the plate (Fig. 1). No differences were noted between the two dose–response curves obtained under these conditions, indicating that there was no matrix interference. Unlike the control human serum samples, whole blood haemoly sate spiked with 4 LD$_{50}$ botulinum toxin ml$^{-1}$ had a slight inhibitory effect (up to 40 % at a 1 in 4 sample dilution) on the signal intensity generated by the toxin. This was due to the formation of a precipitate after initial dilution with sample buffer. A brief (10 min) centrifugation step before further diluting the supernatant on a dual-coated plate eliminated this inhibitory effect (Fig. 2). No false positives were seen with normal serum or whole human blood haemolysate (95/528, NIBSC, UK) controls.

Table 2. Environmental sample extracts for testing in the FDC assay

<table>
<thead>
<tr>
<th>Sample details*</th>
<th>MBA</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food remnant (chicken and korma sauce) extract</td>
<td>Positive for type A toxin</td>
<td>2011</td>
</tr>
<tr>
<td>Pita bread extract</td>
<td>Negative</td>
<td>2011</td>
</tr>
<tr>
<td>Houmous extract</td>
<td>Negative</td>
<td>2011</td>
</tr>
</tbody>
</table>

*Environmental food extracts from the 2011 UK botulism outbreak (Browning et al., 2011).
Sensitivity and specificity of the FDC assay

Assay sensitivity was obtained by calculating the background mean plus 3 SD ($n=19$), utilizing data shown in Fig. 1. The assay limit of detection was 0.03 LD$_{50}$ per ml serum (or 0.13 pg per ml serum). Assay specificity was tested by spiking the control serum with the six botulinum toxin serotypes B to G. No cross-reactivity was seen, indicating that the test is specific for type A toxin alone (Fig. 3).

Measurement of toxin levels in clinical human serum samples using the FDC assay

The FDC assay was applied to quantify toxin levels in clinical sera (Fig. 4, Table 3). The dose–response curve for the most positive serum (S4) taken from the first patient admitted to hospital is given (Fig. 4). Similar shaped dilution curves were obtained from both the haemolysed and non-haemolysed samples.
clinical samples and the spiked reference serum, indicating that the haemoglobin present had no inhibitory effect on the assay. Toxin levels in the three haemolysed serum samples obtained from the first two UK patients (S4, S5, S6) were quantified and each found to contain just 1 to 2 LD₅₀ per ml serum (Table 3). A small washout volume of the remaining unlysed serum samples taken from the same type A botulism patients which had been diluted (>1 in 3) in PBS also tested positive for type A toxin in the FDC assay (data not shown). As expected, three serum samples (S1, S2, S3) tested negative for botulinum type A toxin (LoD 0.03 LD₅₀ per ml serum). Two of these (S1, S2), which previously tested positive for type B toxin in the MBA, were from a type B botulism outbreak in Austria (Vossen et al., 2012), while the other sample (S3) was from a non-botulism case. This confirmed the FDC assay’s specificity for type A toxin using clinically relevant samples.

**DISCUSSION**

Botulinum neurotoxin is considered the most potent known toxin. Spores of *Clostridium botulinum* producing type A toxin are particularly resistant to heat inactivation, and exposure for 25 min at 100 °C is required to kill 90% of the organisms (McLauchlin et al., 2006). Perhaps as a consequence, type A toxin accounts for a large proportion of botulism cases, including a recent outbreak in the UK which resulted from the consumption of contaminated korma sauce (Browning et al., 2011). Of the family members afflicted by the incident in 2011, two individuals were severely affected by botulism. Symptoms rapidly progressed to respiratory paralysis, and mechanical ventilation and antitoxin were required to stabilize their conditions. Some of the initial serum samples, taken soon after admission to hospital, were severely haemolysed and, as a result, could not be confirmed in the MBA (Wagener et al., 2001; ASM, 2003; Rother et al., 2005). Even when unlysed serum samples were obtained, the toxin could not be detected in the third botulism case, which also required antitoxin (Browning et al., 2011). The insufficient sensitivity of the MBA in detecting botulinum toxin in human serum samples is well documented, with levels being frequently undetectable (Woodruff et al., 1992; McLauchlin et al., 2006; Wheeler et al., 2009), but capable of inducing full-blown botulism and respiratory paralysis in patients. This is further supported by experimental data in non-human primates, in which serum levels of type A toxin could not be detected using the MBA (LoD 2 LD₅₀ per ml serum) during a lethal aerosol challenge (Sanford et al., 2010).

**Table 3.** Quantification of type A toxin levels in clinical serum samples using the modified FDC assay

<table>
<thead>
<tr>
<th>Botulism diagnosis</th>
<th>Serum sample code</th>
<th>FDC assay (LD₅₀ per ml serum with 95 % CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A</td>
<td>S4</td>
<td>2.01 (1.57–2.57)*</td>
</tr>
<tr>
<td>Type A</td>
<td>S5</td>
<td>1.15 (1.03–1.29)*</td>
</tr>
<tr>
<td>Type A</td>
<td>S6</td>
<td>1.31 (1.18–1.47)*</td>
</tr>
<tr>
<td>Type B</td>
<td>S1</td>
<td>Negative</td>
</tr>
<tr>
<td>Type B</td>
<td>S2</td>
<td>Negative</td>
</tr>
<tr>
<td>Non-botulism patient</td>
<td>S3</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Activity determined by parallel line analysis relative to a reference (control serum spiked with a known concentration of type A toxin).

**Table 4.** Quantification of type A toxin levels in environmental food extract samples using the modified FDC assay

<table>
<thead>
<tr>
<th>Food sample extract</th>
<th>FDC assay (LD₅₀ per ml extract with 95 % CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food remnant (chicken and korma sauce)</td>
<td>564.97 (467.29–682.59)*</td>
</tr>
<tr>
<td>Pita bread</td>
<td>0.32 (0.27–0.38)*</td>
</tr>
<tr>
<td>Houmous</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Activity determined by parallel line analysis relative to a reference (control serum spiked with a known concentration of type A toxin).
To address these limitations, a new FDC assay was devised. The modified assay procedure entails coating plates with SNAP251–206 substrate plus high-affinity antibody specific for the toxin’s H\textsubscript{C} domain. Toxin capture via the H\textsubscript{C} domain in the presence of milk and enzyme inhibitor is then performed and any interfering sample matrix constituents washed away. The simple addition of an enzymically optimal buffer reduces the critical disulfide bond joining the H- and L-chains of captured toxin molecules and releases the activated L-chain, which then cleaves the immobilized SNAP251–206 substrate. Cleaved SNAP251–197 is subsequently quantified using a neoepitope antibody specific for the newly exposed Q\textsuperscript{197} epitope. The modifications allowed both lysed and unlysed serum samples to be tested for the first time whilst retaining an assay sensitivity down to 0.03 LD\textsubscript{50} per ml serum (~70 times greater sensitivity than the conventional mouse assay). All the provided serum samples from the hospital admissions could be tested by the new serotype-specific assay and toxin levels of 1 to 2 LD\textsubscript{50} ml\textsuperscript{-1} serum were detected, confirming the clinical diagnosis and treatment for type A botulism. Together with its excellent sensitivity, the assay required just 100 µl sample per test (1/5 of the sample volume needed for MBA), which is of considerable benefit when only small volumes of clinical material are available. Other assays are reported to lack the desired sensitivity (Akbulut et al., 2005; Wilder-Kofie et al., 2011; Joshi, 2012) to replace the MBA. For instance, the conventional ELISA test is less sensitive than the MBA and cross-reactivity between serotypes can be problematic (Wilder-Kofie et al., 2011). Even a more recently described high-affinity monoclonal antibody capture ELISA was seven times less sensitive than the modified FDC assay (Zhang et al., 2012) while a BoTest immunoprecipitation method was no more sensitive (LoD 0.1 pM, ~3 LD\textsubscript{50} per ml serum) than the MBA (Dunning et al., 2012). Additionally, sensitive (LoD 0.01 LD\textsubscript{50} ml\textsuperscript{-1}) direct endopeptidase tests (Jones et al., 2008) are adversely affected when partially or undiluted serum is used (R. G. A. Jones, unpublished data; Capek & Dickerson, 2010).

Botulinum neurotoxins are naturally complexed with one or more non-toxic neurotoxin-associated proteins (NAPs). NAPs are important to toxicity by ingestion in that they protect the neurotoxin from acidity and proteases in the stomach (Maksymowych et al., 1999; Gu et al., 2012). Although tightly associated under acidic conditions, the NAPs rapidly dissociate from the dachain toxin molecule at neutral pH and, as a result, the toxin is only found as a complex-free form in the slightly alkaline (pH 7.4) conditions of the blood (Eisele et al., 2011). During the FDC assay, the capture step is performed in a slightly alkaline (pH 7.4) CP-buffer, which ensures the dissociation of any complexed molecules that may be present in food extracts or low-pH samples. In environmental food extracts, toxin levels previously detected by MBA were accurately quantified by the new FDC assay. A sample of pita bread extract, which previously tested negative in the MBA, gave a value of just 0.32 LD\textsubscript{50} of type A toxin per ml food extract when assessed by the FDC assay, perhaps indicating that the original pita bread sample recovered from the rubbish had become contaminated with traces of korma sauce.

Using previously coated plates, each FDC assay took approximately 27 h to complete. Further optimization, however, would easily reduce the time required or increase the sensitivity, if necessary, making the assay more suited to routine use. For instance, the use of a high-performance ELISA microplate incubator/shaker would allow major reductions in incubation time during several steps (Mushens & Scott, 1990). The PIC concentration could also be reduced when testing clinical serum samples. The direct conjugation of HRP to the neoepitope antibody could potentially remove one step of the assay. Additionally, performing the SNAP251–206 cleavage step at a constant 23 °C optimal temperature and using a different signal detection system with greater amplification would increase sensitivity and reduce assay time.

The high sensitivity of the modified FDC assay also has the potential to enable better in vivo pharmacokinetic studies in the future when sublethal dosages of biologically active toxin are utilized. Previous studies failed to detect toxin in the blood compartment or relied on the use of modified radio-labelled toxin that was partly or fully inactive (Tang-Liu et al., 2003; Ravichandran et al., 2006; Sanford et al., 2010). The FDC assay provides an important improvement by allowing a more accurate protracted measurement of blood toxin levels. The application of mass spectrometry combined with the use of specific monoclonal-antibody-coated beads has been developed for potential use with clinical or pharmacokinetic samples. This technique, however, is 12 times less sensitive than the FDC assay described here (LoD 0.36 LD\textsubscript{50} per ml serum), uses 500 µl serum per test and takes 24 to 48 h to perform (Parks et al., 2011). A more recently described immunocapture mass spectrometry method is no more sensitive than the MBA (Mazuet et al., 2012). The costs of the specialized equipment used in mass spectrometry may also be prohibitive for most public health laboratories (Wilder-Kofie et al., 2011). A promising new experimental approach which uses ultrabright fluorescent supra-nanoparticles to amplify the detection signal has been published recently by Bok et al. (2013). However, the assay has yet to be tested in a serum matrix, and the use of antibodies known to cross-react with other toxin serotypes is likely to limit its application in serotype identification.

Unlike the assays described by Evans et al. (2009) and Behrensdorf-Nicol et al. (2010), which also quantify the H\textsubscript{C} domain with linked L-chain endopeptidase activity, the FDC assay is performed in a single dual-coated well, which makes it much easier to perform. Additionally, it does not require isolated rat brain synaptosomes, as in the assay of Evans et al. (2009). Bagramyan et al. (2008) described a highly sensitive, rapid, bead-based ALISSA test that also incorporates an initial toxin capture step utilizing an...
anti-botulinum toxin A antibody followed by the endopeptidase cleavage of SNAP25 by the L-chain (Bagramyan et al., 2008; Bagramyan & Kalkum, 2011). Despite being described 5 years ago, the ALISSA test has not been widely adopted by other laboratories, or applied to real clinical samples, perhaps as a consequence of its high reagent costs, which amount to $14.94 per well of a 96-well plate (Bagramyan et al., 2008). Limitations of other assay formats, such as the liposome PCR (Mason et al., 2006a, b), are reviewed elsewhere (Capek & Dickerson, 2010).

That no specialized equipment is required to perform the FDC assay other than conventional ELISA apparatus, which is currently utilized by most public health laboratories, is another major benefit. Reagent costs for the FDC assay are also similar to those for a conventional capture ELISA plus the additional costs of recombinant human SNAP251–206 (£0.13 per well), PIC (£0.10 per well) and DTT (£0.01 per well). The anti-HC capture monoclonal antibody was produced in-house (3D12, University of California); however, with an ever-increasing number of commercially available antibodies, it may be possible to utilize a suitable alternative. Rabbit polyclonal antibody (anti-SNAP25197) specific for the neoepitope generated by the specific cleavage of SNAP25 with type A toxin has been produced successfully in various laboratories or custom generated (Hallis et al., 1996; Fernández-Salas et al., 2004; Liu et al., 2012). It may, however, be possible to substitute this with a commercially available mouse monoclonal antibody from Stratech Scientific (USBiological, S1014-73D) or Research & Diagnostic Antibodies (MC-6053) at a cost of £0.07 per well, providing that the subsequent goat anti-rabbit HRP conjugate step is replaced by a suitably specific anti-mouse material. That the FDC assay is performed on an inexpensive, dual-coated single surface also lends itself to future use on a chip and the rapidly developing field of microfluidic technology and automation (Capek & Dickerson, 2010; Lillehoj et al., 2010; Weingart et al., 2012).

In summary, we describe here for the first time the development and application of a modified highly sensitive FDC assay which positively identifies and specifically quantifies low levels of botulinum type A toxin in serum and food samples. The new assay provides a real alternative for public health laboratories to unambiguously confirm all cases of type A botulism.

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