Development and comparison of two immunoassay formats for rapid detection of botulinum neurotoxin type A

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Abstract

We have evaluated two formats of immunoassays for the rapid detection of *Clostridium botulinum* neurotoxin type A (BoNT/A), in assay buffer and various matrices (human serum and nasal swabs, fresh milk, sugar, flour and talcum). The two formats, a vertical-flow strip immunochromatography (ICT) and a small disposable immunoaffinity column (IAC), were selected because they are both rapid and readily usable in the field without sophisticated equipment. We utilised the same critical reagents to develop and optimise both assays, making it possible to compare the corresponding technologies on the same toxin preparations, without interference due to the properties of the antibodies. Results were interpreted using a standard statistical test (ANOVA) and showed little difference of sensitivity between matrices. Though both assays were completed in 40 min, the sensitivity of the IAC, evaluated at 0.45 pM (5 mouse LD50 units/ml), was 40 to 80 times better than that of the ICT. Furthermore, the sensitivity of the IAC assay was improved to 0.09 pM (1 mouse LD50 unit/ml) when performed on a 5-ml volume of human serum. Thus, the IAC appears to be one of the most sensitive and rapid assays for the detection of BoNT/A reported to date and, because it is also highly transportable, it is amongst the best suited for field diagnosis of BoNT/A poisoning.

Keywords: Botulinum neurotoxin; Rapid detection; Antibody-based assays; Immunochromatography; Immunoaffinity column

1. Introduction

Natural human botulism occurring through accidental food poisoning or inoculation by drug abusers became an infrequent disease, though it is not disappearing (Sobel et al., 2004; Lindstrom and Korkeala, 2006). The main concern about botulinum neurotoxins (BoNTs) has now been focused on their potential use as bioweapons because they are relatively easy to produce, are highly potent and lethal, and the induced paralysis requires prolonged intensive care (Arnon et al., 2001).

BoNTs are 150,000 Da proteins with zinc-endopeptidase activity, secreted by the bacterium *Clostridium botulinum* as complexes with non-toxic proteins. The neurotoxicity requires that the BoNT reaches the cytoplasm of presynaptic nerve endings where its enzymatic activity results in the cleavage of proteins involved in vesicle docking, thus inhibiting neurotransmitter release. There are seven defined serotypes (designated A to G) of BoNTs. Type A (BoNT/A) is one of the most frequent causes of natural human botulism, is implicated in the most severe cases, and is the serotype probably presenting the highest threat as a bioweapon (Arnon et al., 2001).

Abbreviations: BoNT/A; botulinum neurotoxin type A; ICT; strip immunochromatography; IAC; immunoaffinity column; LD50; 50% lethal dose; poly-HRP; polymerized horseradish peroxydase.

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BoNTs must be detected rapidly to enable timely administration of neutralizing antibodies to the exposed persons and to prevent additional exposures. A debated study recently analysed the risk of bioterrorism involving the release of BoNT into milk supplies (Wein and Liu, 2005). The findings stressed the need for assays that could be used in the field and that allow rapid detection of BoNTs.

The reference method for detecting BoNTs continues to be the mouse lethality assay because it is a highly sensitive functional assay that directly measures the amount of toxic BoNT. Nevertheless, it has well-known limitations. Foremost, this assay usually requires 24 to 48 h to yield results. However, antibody therapy is most efficient when given before the onset of clinical signs of botulism, which can occur less than 12 h after exposure. Also, the necessity to maintain test mice makes it impractical outside reference laboratories and particularly in field conditions.

Various assays for BoNTs have been developed, aiming to equal the sensitivity of the mouse lethality assay while alleviating its limitations (Doellgast et al., 1993; Ekong et al., 1995; Szilagyi et al., 2000; Schmidt et al., 2001; Chiao et al., 2004; Barr et al., 2005; Gessler et al., 2005; Sapsford et al., 2005; Sharma et al., 2005, 2006; Rivera et al., 2006). Antibody-based assays have been the most studied. These differ in procedure time and sensitivity due to the assay design and the technology used to produce the detection signal. It is possible to compare the performances of various immunoassay formats only if the same antibodies are used for each, because the sensitivity of the assay depends largely on the affinity of the antibodies for the target antigen. We recently used this approach to compare ELISA and electrochemiluminescence immunoassay technologies for the detection of BoNT type B (BoNT/B) (Guglielmo-Viret et al., 2005).

In the present study, we evaluated two formats of immunoassay for the rapid detection of BoNT/A. One was a vertical-flow strip immunochromatography (ICT) with colloidal gold particles, and the other was a small disposable immunoaffinity column (IAC) with a polymerized form of peroxidase as the detector. We selected these formats because they yield rapid results and can be used in the field without sophisticated equipment. Although they have been used previously for BoNT detection (Chiao et al., 2004; Gessler et al., 2005), the two formats have not been compared directly. Here, we used the same antibodies to develop and optimise both formats and compared their performance on the same toxin preparations, in assay buffer and various matrices. Development and use of point-of-care and field-portable assays are increasing, thus such a comparison should be of wide interest.

2. Materials and methods

2.1. Botulinum toxins

Purified BoNT complexes, derived from type A1 Hall, type B Okra, and type E Alaska strains were purchased from Metabiologics Inc. (Madison, WI, USA). The BoNT complex preparations were indicated by the manufacturer to be pure (by SDS-PAGE) and have specific activities of 2.2×10^7 mouse 50% lethal dose (mouse LD50 unit)/mg for BoNT/A, 1×10^7 mouse LD50 unit/mg for BoNT/B, and 3×10^7 mouse LD50 unit/mg for BoNT/E complexes. An average molecular weight of 500,000 Da was used to calculate the molar concentration of the BoNT/A complex (Inoue et al., 1996; Smith et al., 2005). Aliquots of the preparations (0.5 mg/ml in 50 mM citrate buffer pH 5.5 containing 5% BSA) were stored at −80 °C, and used immediately after thawing. The mouse lethality assay was used as described (Solomon and Lilly, 2001) to verify the specific activity of one freshly thawed aliquot for each serotype. Briefly, Swiss (RjOrl) mice (Elevage Janvier, Le Genest Saint Isle, France) weighing 18 to 22 g were injected with serial two-fold dilutions of toxin, based on the specific activity values provided by Metabiologics, in gelatin–phosphate buffer (50 mM NaH_2PO_4, 0.2% gelatin, pH 6.2). A total of 0.5 ml was injected intraperitoneally into each of two mice for each dilution. Animals were monitored for 72 h post-injection and examined regularly according to institutional guidelines. The specific activities of the thawed aliquots were found to be identical to those determined by the manufacturer.

2.2. Antibodies

Two antibodies were used in this study. One was an affinity-purified rabbit polyclonal IgG (rabbit pAb) raised against purified BoNT/A (Metabiologics Inc.). The other one (mAb 11F7) was a monoclonal murine IgG 2a, derived by hybridoma technology from a mouse immunized against the 50 kDa C-terminal fragment of the BoNT/A (subtype A1) heavy chain, that was purified from a recombinant *E. coli* strain generously provided by Dr. G. Schiavo (ICRF, London, UK) (Lalli et al., 1999). The 11F7 mAb was purified on a Hi-Trap protein A HP Column (GE Healthcare/Amersham Biosciences, Orsay, France), according to the manufacturer's instructions.

2.3. Vertical-flow strip immunochromatography test (ICT)

The principle and design of this assay have been described formerly (Guglielmo-Viret et al., 2007). An initial series of ICTs was made under fixed conditions. The capture antibody (0.5 μg in PBS/test) was sprayed at a rate of 1 μl/cm to form a capture line on polyester-backed nitrocellulose membrane (MTP, Whatman, Kent, UK) with a dispenser (IsoFlow reagent dispenser, Biodot, Chichester, UK). A control line made of Goldline (British Biocell International, Cardiff, UK), which chemically reacts with gold, was applied 8 mm above the capture line. The detection antibody (0.1 μg/test) conjugated to anti-mouse or anti-rabbit IgG gold beads (40 nm diameter, British Biocell International) (5 μl beads at $OD_{520}=10$ per test) was sprayed onto a 1.7 mm-wide conjugate pad (British Biocell International) at a flow rate of 15 μl/cm. After application of the reagent, the nitrocellulose membrane and conjugate pads were dried for 2 h at 37 °C, then assembled with the absorbent pad (Whatman) and the sample pad (British Biocell International) using the sticky backing of the MTP. The assembled strips were cut to a width of 5 mm using a Guillotine cutter (BioDot).

Under these conditions, we compared two set-ups using either the rabbit pAb for capture and 11F7 mAb for detection, or vice versa. The assays were run with 100 μl of BoNT/A complex (with concentrations from 10 to 0.1 μg/ml) spiked in PBS-Tween 0.5%-BSA 0.5%, and this volume practically represents a maximum for an adequate migration. The best set-up was chosen and various conditions were tested for further optimisation: the capture antibody (0.5 to 2 mg/ml) in PBS with BSA (0 to 2%) was sprayed in volumes from 0.5 to 2 μl/test. The detection antibody (5 to 50 μg/ml) was conjugated with various concentrations of gold beads ($OD_{520}=1$ to 10) before spraying. The assay buffer was composed of PBS, Tween (0 to 1%) and BSA (0 to 2%) and was used at a volume of 100 μl/test. Before use, strips were stored at 4 °C in a hermetic tube with a desiccant packet.

The limit of detection apparent by the naked eye was determined by trained personnel, as would be the case in the field. The strips were also read by a GelDoc XR imaging system (Biorad, Hercules, USA) and the associated software, Quantity One 4.6.0, to ensure reproducibility of detection and to quantify the signal. First, bands were automatically detected to confirm the naked eye detection. Second, bands were manually delineated and the intensity of the signals was calculated as the mean intensity of the pixels inside the surface of the band minus that of background (same surface manually defined in the vicinity of the band), using a fixed exposure time setting of 0.045 s.

2.4. Immunoaffinity column (IAC) assay

In this assay the sample and reagents are passed over a miniature disposable plastic column (65 mm × 7 mm) packed with a 5 mm × 5 mm polyethylene filter (average pore size of 20 μm) at the bottom. The filter, coated with a capture antibody, traps the analyte which is then detected by the binding of a biotinylated antibody, followed by streptavidin conjugated to a polymerized form of horseradish peroxydase (streptavidin-poly-HRP) (Fig. 1).

This technology is patented and commercialised under the name Abicap®, by Senova GmbH (Jena, Germany) which produced and optimized the test components. The signal produced in the column is directly quantified with a small (20 × 10 × 4 cm) dedicated photometer (Abicap reader, Senova). We decided to use the mAb 11F7 as the capture antibody because of the relatively large amounts requested by Senova for coating filters (10 mg). Briefly, filters were coated by adsorption with mAb 11F7 diluted in carbonate buffer (100 mM Na2CO3, 100 mM NaHCO3, pH 9.5) at a concentration of 15 μg/ml for 40 min at room temperature and then saturated with 750 μl PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.05% 5-bromo-5-nitro-1,3-dioxane as a preservative. The columns were stored in plastic blisters under vacuum at room temperature. The rabbit pAb (detection antibody) was biotinylated with Sulfo-NHS-LC Biotin (Pierce, Perbio Science, Germany) at a biotin-to-antibody molar ratio of 20:1. Biotinylation was performed according to the manufacturer's instructions, which included the removal of unconjugated biotin by dialysis.

We modified and tested various parameters for optimisation of the procedure. These included the concentration of detection antibody (0.5–10 μg/ml), the sequential application of sample and detection antibody to the column or their pre-incubation, the time and temperature of this pre-incubation (10 min at room temperature or 1 h at 37 °C), the degree of polymerisation of the HRP conjugated to streptavidin (poly-HRP 40 or poly-HRP 80), number of washes and composition of wash buffer (PBS-BSA 0.2%, PBS-Tween 0.1%). The optimisation led to the selection of the following protocol. Reagents were added sequentially to each test column, and let flow through by gravity, in the following order : 750 μl wash buffer (WB: PBS-BSA 0.2%), a volume of sample as indicated, 500 μl WB, 500 μl detection antibody (2.5 μg/ml in PBS-casein 2.5%), 500 μl WB, 500 μl streptavidin-

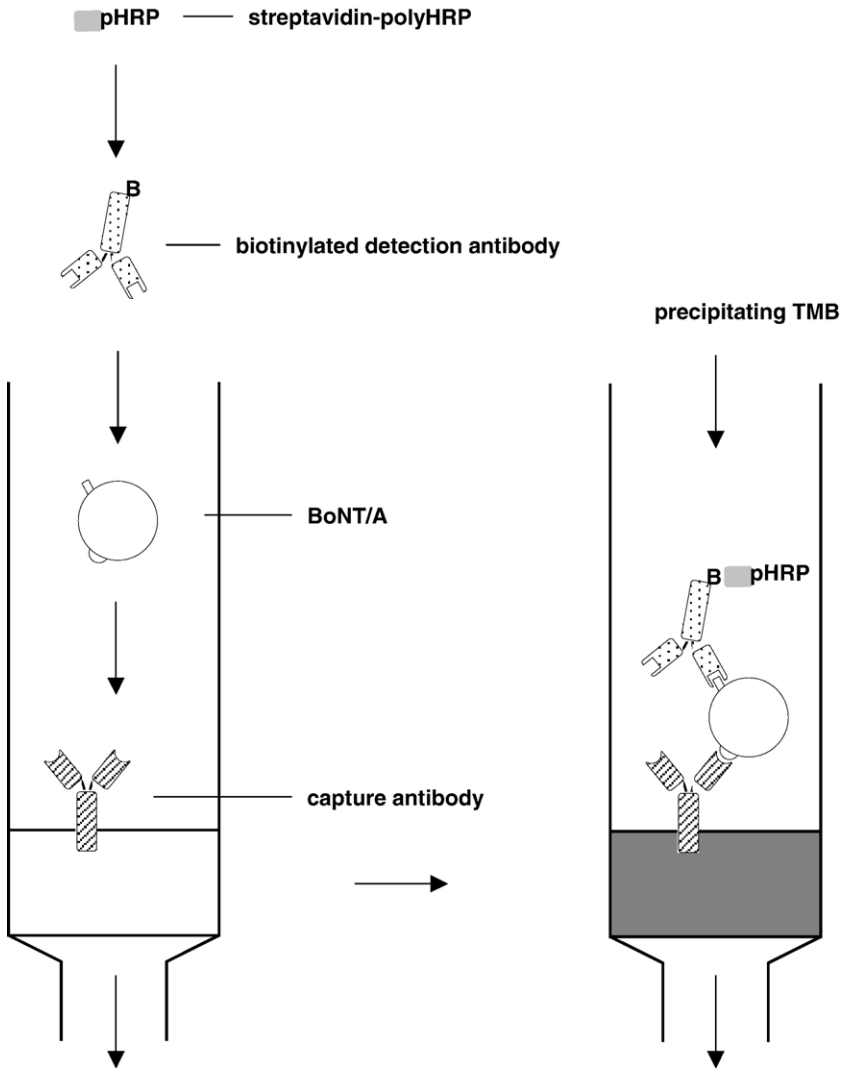

Fig. 1. IAC assay set-up. The sample is passed over the column by gravity. A capture anti-BoNT/A antibody coated on the filter binds the toxin present in the sample. Captured toxin is detected by the sequential application of biotinylated anti-BoNT/A antibody, Streptavidin-poly-HRP and precipitating TMB, resulting in a blue coloration of the filter. Each step is followed by one wash as indicated in the text.

poly-HRP 40 (1 mg/ml), 500 µl WB, 500 µl substrate buffer (0.1 M Na-Acetate-Citrate-Buffer, pH 4.9), 500 µl precipitating HRP substrate (TMB), and again 500 µl substrate buffer. The absorbance was measured directly on the column. The sample, detection-antibody, poly-HRP and TMB steps each included a 6-min incubation. The whole procedure was performed at room temperature.

2.5. Statistics

All samples were tested in triplicate. We used one-way ANOVA followed by a multiple comparisons versus control group test (Holm–Sidak method) (SigmaStat 3.0; SPSS Inc., Chicago, IL, USA) for analysis of each IAC series of measurements. The limit of detection was defined as the smallest concentration of BoNT/A complex giving a signal significantly different ($p < 0.05$) from the negative control. For IAC and ICT, coefficients of variation (CVs) were calculated as ratios of the standard deviations to the means at the respective concentrations of BoNT. Standard curves were fitted by non-linear regression using a four-parameter logistic equation (Prism version 5, GraphPad Software Inc., San Diego, CA, USA).

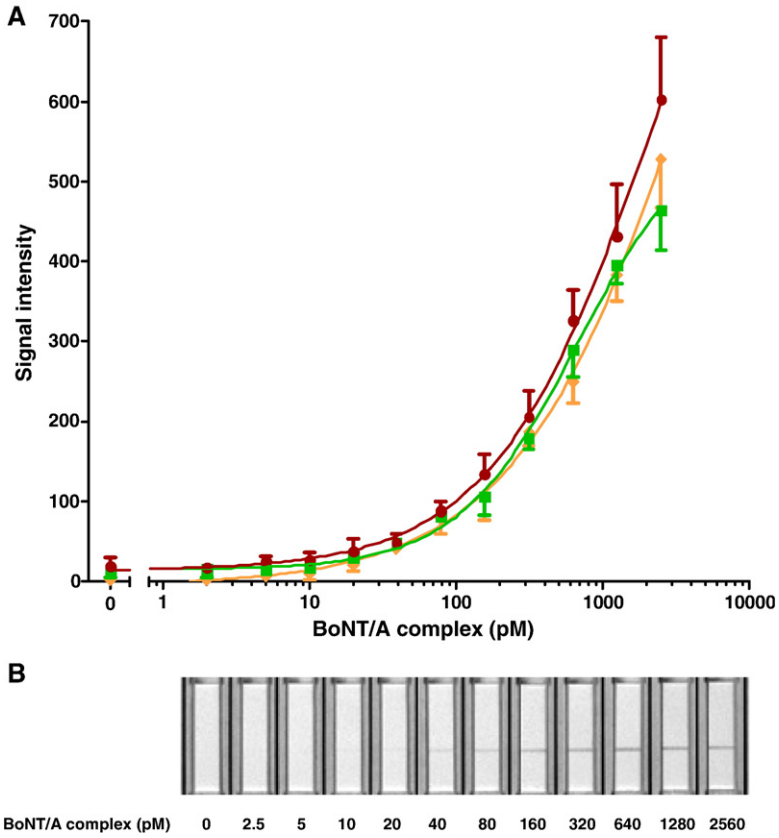

Fig. 2. ICT assay. (A) Calibration curves for BoNT/A complex in assay buffer. Points represent the mean signal intensities calculated as indicated in Materials and methods and expressed as a function of BoNT/A concentration, for triplicate strips on each day. The experiment was repeated on three days. Standard deviations are indicated as error bars. Where not visible, the error bars fall within the symbol itself. (B) Photograph of representative capture line signals for various concentrations of BoNT/A complex. The limit of detection is 20 to 40 pM.

2.6. Sample preparation in selected matrices

Human serum (pooled donors) was purchased from Sigma Chemical (St Louis, MO, USA). Fresh whole milk, sugar, flour and talcum were purchased from a local supermarket. Nasal sampling was performed on laboratory personal using a standard bacteriology swab (Eurotubo Collection swab, I.A.S.A., Rubi, Spain) per nostril, each washed in 1 ml of assay buffer. Flour, sugar and talcum were dissolved or suspended (5% w/v) in assay buffer. The two powders represent nutrient compounds while talcum mimics dust. Milk and serum were centrifuged at 13,000*g* for 15 min at 4 °C, then the lipid layer was discarded and the aqueous supernatants were spiked with the various concentrations of BoNTs. For ICT, but not for IAC, serum and milk samples were

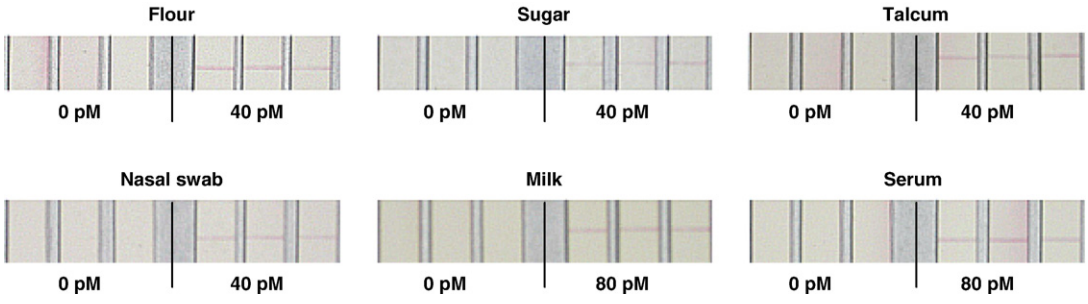

Fig. 3. Limits of detection with the ICT assay in various matrices. The matrices were spiked with the indicated concentrations of BoNT/A complex; the assays were realized in triplicates. Photographs of corresponding capture line signals.

diluted with equal volumes of assay buffer prior to testing. For IAC on human serum, Super ChemiBlock heterophile blocking agent (Chemicon International Ltd, Chandlers Ford, UK) was added to the samples at a final concentration of 50 µg/ml.

3. Results

3.1. ICT

The ICT was best prepared with the rabbit pAb used for capture and the 11F7 mAb used for detection. Conditions for manufacturing were optimized as follows. The capture antibody (1 mg/ml) in PBS was applied at a flow rate of 1 µl/cm membrane. The detection antibody (final concentration of 15 µg/ml) was incubated (2 h at 37 °C) with anti-mouse IgG gold beads (OD_{520}=7) before application at a flow rate of 15 µl/cm. After drying (2 h at 37 °C), the coated membranes were assembled with the absorbent pad and the sample pad, then cut in 5 mm-wide strips. Assay buffer was composed of PBS and Tween (0.5%) and was used at a volume of 100 µl/test. The test protocol, including drying, took about 40 min. Samples spiked with various concentrations of BoNT/A complex, from 2 to 2500 picomolar (pM), had detectable signals starting

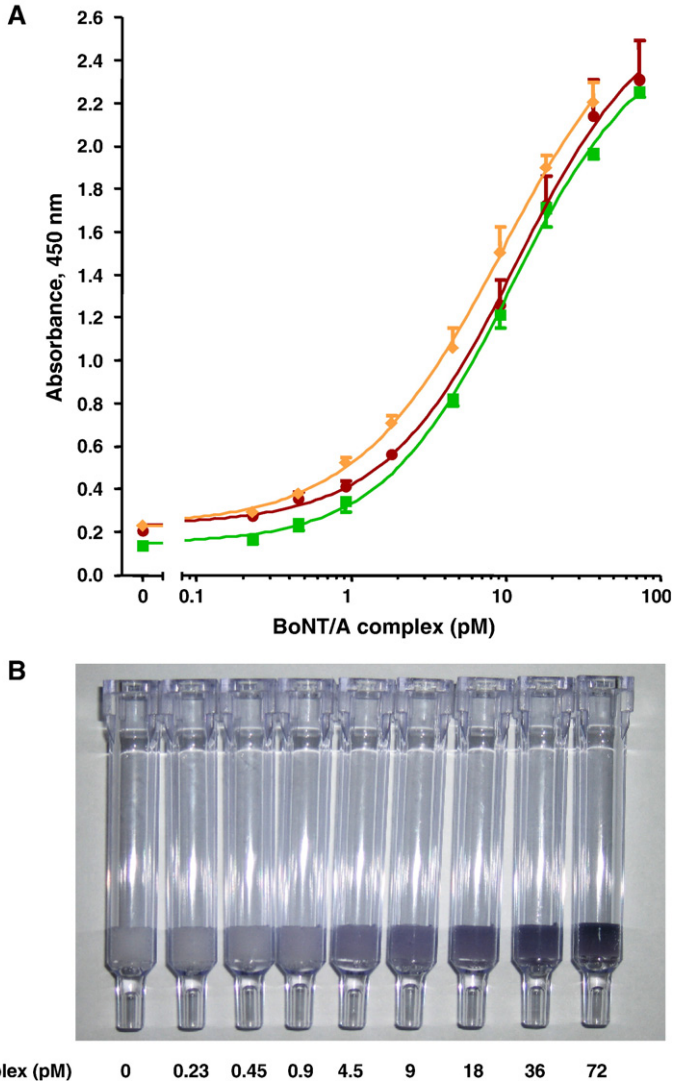

Fig. 4. IAC assay. (A) Calibration curves for BoNT/A complex in assay buffer, with sample volumes of 750 µl. Points represent the mean absorbance values expressed as a function of BoNT/A concentration, for triplicate columns on each day. The experiment was repeated on three days. Standard deviations are indicated as error bars. Where not visible, the error bars fall within the symbol itself. (B) Representative columns for various concentrations of BoNT/A complex. The limit of detection by photometry is 0.45 pM and by the naked eye is 4.5 pM.

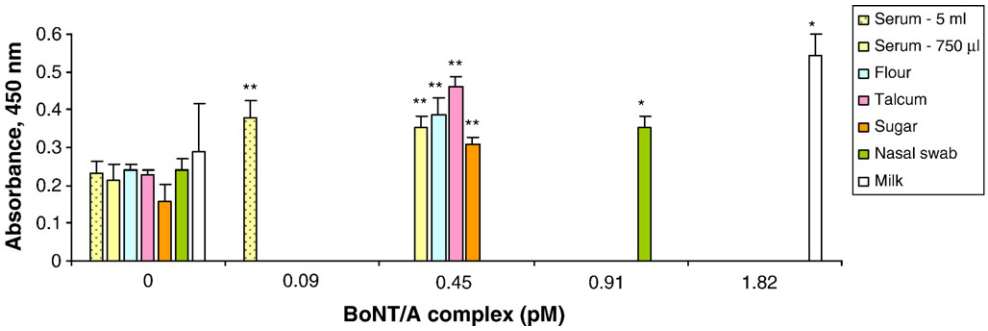

Fig. 5. Limits of detection with the IAC assay in various matrices. The matrices were spiked with the indicated concentrations of BoNT/A complex; the assays were realized in triplicates. Where not indicated, the sample volume was 750 µl. Error bars and asterisks indicate standard deviations and statistical significance (*$p<0.05$, **$p<0.01$), respectively.

with 20 to 40 pM (220 to 440 mouse LD50 unit/ml), which corresponds with the limit of detection established both by the naked eye and the imaging system (Fig. 2). These measurements were repeated for three days, the intra-day CV (evaluating reproducibility) was 17.5% for the whole range of concentrations tested. The CV between days (evaluating repeatability) was 18.6%. Six months storage at 4 °C in the presence of a desiccant did not affect the detection limit of the strips.

No visible signal was obtained in samples of PBS-Tween 0.5% spiked with BoNT complex type B or type E at 200 pM.

The sensitivity of the ICT assay was also evaluated in matrices other than buffer (Fig. 3). For all tested matrices except serum and milk, concentrations of 40 pM gave readable signals while concentrations of 20 pM gave very weak signals. For serum and milk, BoNT/A complexes were spiked at 80 pM, then diluted prior testing, corresponding to a final concentration of 40 pM in the mobile phase as for the other matrices and this concentration gave readable signal. No false positive was observed in any of the tested matrices and all samples were assayed in triplicates. No significant loss of sensitivity was thus observed in the selected matrices, except for a two-fold loss in serum and milk, corresponding to the dilution in assay buffer necessary to ensure their migrations.

3.2. IAC assay

The selected protocol was completed in about 40 min for a sample of 750 µl, the volume recommended by the manufacturer. In assay buffer spiked with various concentrations of BoNT/A complex (0.09 to 900 pM) the limit of detection was at 0.45 pM (5 mouse LD50 unit/ml) ($p<0.01$) and the signals became higher with higher concentrations until a plateau was reached with about 80 pM BoNT/A complex (Fig. 4). The columns became clogged if 900 pM BoNT/A complex was used, presumably due to a large amount of TMB precipitating in the filter. These measurements were repeated for three days, the intra-day CV (evaluating reproducibility) was 6.3% for the whole range of concentrations tested. The CV between days (evaluating repeatability) was 14.3%.

We observed no cross reactivity for samples spiked with BoNT complex type B or type E at 200 pM. Storage for 12 months at room temperature did not affect the detection limit of the columns.

The sensitivity of the IAC assay was also evaluated in matrices other than buffer (Fig. 5). For sample volumes of 750 µl, the limit of detection for BoNT/A complex remained unaffected at 0.45 pM ($p<0.01$) in all tested matrices, except for nasal swab and fresh milk where it was at 0.91 pM ($p=0.01$) and 1.82 pM ($p=0.03$), respectively.

For human serum we tested whether the analysis of larger volumes of sample would lower the limit of detection and found that increasing the volume to 5 ml allowed the sensitivity to reach a value of 0.09 pM (1 mouse LD50 unit/ml) ($p<0.01$). In that case the procedure was longer, due to the larger volume of sample to flow through, but was completed in less than an hour.

4. Discussion

In this study we used commercially sourced BoNT/A complex as the analyte to compare the capability, especially sensitivity of two rapid immunoassay formats, ICT and IAC. We initially ran the assays in standard buffer to avoid effects from sample matrices and facilitate comparison with previously published results. We then evaluated the effect of various matrices on the sensitivity of both assays. We used the BoNT complex in this study because it is the most stable form of the

botulinum neurotoxin and the form that would be encountered in most types of samples. Other reported assays have sometimes been evaluated with purified neurotoxin instead of BoNT complex. A ratio of one molecule of neurotoxin (MW 150,000 Da) per molecule of complex (average MW of 500,000 Da) can be assumed (Inoue et al., 1996), thus equivalences can be calculated. We expressed the limits of detection as molar concentrations of BoNT, both in our results and in the discussion below, again to facilitate comparison of values obtained with the two forms of toxin.

The limit of detection for the various assays must be compared to that of the mouse lethality assay, the gold standard, which is expressed in toxicity concentrations such as a number of mouse LD50 unit per ml of sample. However non-functional assays including immunoassays and endopeptidase-activity assays do not measure solely the amount of toxic BoNT. Part of the analyte detected in these assays might not be toxic for motorneurons, due for instance to degradation in regions of the toxin that are responsible for cell binding. Thus a relatively high limit of detection (poor sensitivity) could correspond to a small number of mouse LD50 unit/ml depending on the BoNT preparation used. Therefore it is essential, when evaluating the sensitivity of such assays, that the specific activity of the botulinum toxin preparation is indicated, typically in mouse LD50 unit/mg of toxin, or that reference toxin standards of known toxicity and stability are used (Sesardic et al., 2003). This is particularly important for tests of clinical interest or those that aim to replace the mouse lethality assay.

Both tests evaluated here needed the same amount of time for completion. While the procedure for the ICT is slightly simpler than that for the IAC, the IAC was 40 to 80 times more sensitive. If we had been able to use the affinity-purified rabbit pAb for capture, which we found to be the best set-up during the ICT optimisation, the sensitivity of the IAC might have been even better. The same antibodies were used in both tests, thus the enhanced sensitivity of the IAC is likely due in part to the larger volume of sample that can be analysed (a standard sample volume of 750 µl for the IAC versus 100 µl for the ICT, potentially representing 7.5 times greater sensitivity for the IAC) and to the signal amplification provided by the polymerized HRP.

The intra-day CV was smaller for the IAC (6.3%) than for the ICT (17.5%) but inter-day CVs, that best reflect the consistency of results in practical use, were equivalent (14.3% and 18.6%, respectively). No significant loss of sensitivity was observed in the various matrices except for a two-fold reduction in serum and milk with ICT, corresponding to the dilution necessary to ensure their migrations, and a two-and four-fold reduction in nasal swab and milk, respectively, with IAC. With IAC in serum, it was necessary to add an interfering-antibody blocking agent to keep the background signal at acceptable levels.

Several subtypes of BoNT/A, differing in amino acid sequence by up to 10%, have been recently described and may be recognised with differing affinities by antibodies (Smith et al., 2005; Arndt et al., 2006). If it were the case for the antibodies used in our tests, their sensitivities would have to be evaluated for each subtype.

We evaluated how our assays compare to those reported previously for the detection of BoNT, though they did not use the same antibodies and toxin preparations. Chiao et al. (Chiao et al., 2004) reported an ICT for the detection of BoNT/B with a limit of detection of 330 pM and that could be increased by silver enhancement to 0.33 pM. In our hands, this published protocol for silver enhancement produced high background, resulting in decreased sensitivity. The sensitivity of our ICT, 20 pM (220 mouse LD50 unit/ml), is similar to that reported recently for two commercial BoNT detection kits based on the same technology (Sharma et al., 2005), indicating that our optimisation was efficient.

With a limit of detection of 0.45 pM (5 mouse LD50 unit/ml), that can be lowered to 0.09 pM (1 mouse LD50 unit/ml) when testing 5 ml of serum, our IAC assay is one of the most sensitive rapid assays for detection of BoNT/A reported to date. It approaches the sensitivity of the mouse lethality assay which, for the number of animals usually assayed (Solomon and Lilly, 2001), has a practical limit of detection of 2 mouse LD50 unit/ml. A test based on similar IAC technology for detection of BoNT/C and BoNT/D has been reported (Gessler et al., 2005) with a sensitivity of 1 minimal lethal dose (defined as the minimal dose which kills all mice) per ml. This would correspond with 2 mouse LD50 unit/ml in our experience. Assuming the specific activities of these preparations (in mouse LD50 unit/mg of toxin) were similar to ours, the sensitivities of the two assays (expressed in molar concentrations of BoNT) would be equivalent, indicating that our optimisation was efficient. Our IAC was however more rapid than that of Gessler et al., which required over four h for such sensitivity. Overall, these two studies demonstrate the potential usefulness of the IAC format.

Several amplified ELISA with sensitivities approaching that of the mouse lethality assay for BoNT/A have been reported (Doellgast et al., 1993; Ekong et al., 1995; Szilagyi et al., 2000; Sharma et al., 2006). Though faster than the mouse lethality assay, these ELISA procedures

take about five h in addition to the plate-coating step to yield results. Our IAC assay also offers a favourable compromise of sensitivity and time when compared to other BoNT/A detection assays relying on more sophisticated equipment. These include electrochemiluminescence (Rivera et al., 2006) (0.36 pM, 8 mouse LD50 unit/ml, 2 h), protease activity assays coupled with mass spectrometry (Barr et al., 2005; Kalb et al., 2006) (0.07 pM, 1.2 mouse LD50 unit/ml, 2 h) or coupled with fluorescence detection (Schmidt et al., 2001) (4 pM, 3 h), or array immunosensors (Sapsford et al., 2005) (40 pM of toxoid, 30 min).

Because BoNTs are so toxic, assays for their detection must be highly sensitive if they are to be practical. On closer examination, the required sensitivity depends on the detection situation, thus our two tests, which differ in their sensitivities, may be theoretically of interest in different situations.

Maximum sensitivity is necessary for clinical serum samples because of the dilution factor in the human body. Arnon et al. reported a mildly affected patient (no mechanical ventilation required) whose serum contained 2 mouse LD50 unit of BoNT/A per ml (Arnon et al., 2001). The possibility to analyse large volumes of sample is an asset of the IAC technology, as illustrated by its limit of detection at 0.09 pM (1 mouse LD50 unit/ml) on 5 ml of human serum. Thus, the current sensitivity of the IAC may be sufficient even for the diagnosis of such a mild case.

Inhalation is thought to be the most threatening route for poisoning, at least for military troops as their drinking water and food can be controlled, but protective masks can be worn only for limited periods of time. Diagnosis made possible from analysis of nasal swab samples with one of our tests would be practical for such personnel. A substantial amount of toxin, estimated to be over 10% of the inhaled aerosolised dose (Guglielmo-Viret et al., 2007), would be trapped in the anterior nasal passage. The lethal dose of BoNT for humans by inhalation is not known, but it has been determined to be over 300 mouse LD50 unit/kg in non-human primates (Franz et al., 1993). The extrapolation for a 70-kg patient at this dose suggests that a sample concentration over 210 mouse LD50 unit/ml would be obtained, assuming a 10% recovery of the toxin trapped in the anterior nasal passage by a swab washed in one ml of buffer. The concentration of BoNT obtained by these calculations would be at the low end of the detection limit for the ICT, but could be detected by the IAC with a 20-fold safety margin.

Regarding the oral route, by which only a small fraction of the toxin is absorbed, the lethal dose for humans has been estimated to be 70 μg of BoNT/A (equivalent to 0.5 nmol) (Arnon et al., 2001; Wein and Liu, 2005). If a serving of food or beverage (around 500 ml, for example) contained such an amount of BoNT/A, it would be detected by the ICT, with a 25 to 50-fold safety margin for the given example. This amount of BoNT/A would be detected by the IAC with a 500 to 2000-fold safety margin for this example.

The IAC assay would be more useful than the ICT for the detection of analyte present in minute amounts, as shown in the present study. Although the IAC format is not presently convenient for the analysis of a large number of samples, it does combine essential assets of rapidity, high sensitivity, portability and ease of handling, which are of particular interest for field laboratories. Automation and multiplexing of this technology would be beneficial.

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