Rapid and ultrasensitive detection of botulinum neurotoxin serotype A1 in human serum and urine using single-molecule array method

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Abstract Botulinum neurotoxin (BoNT) is one of the most poisonous substances ever known. It is produced by *Clostridium botulinum*, which causes botulism, a persistent paralysis of peripheral nerve termini. *Clostridium botulinum* bacteria, the source of BoNTs, are widely distributed in the environment. BoNT poses a significant risk as a bioweapon and is classified as a Category A agent by the Centers for Disease Control and Prevention (CDC) because of its ready availability and extreme potency. In this paper, a rapid and ultrasensitive method using a single-molecule array (Simoa) assay was developed for the quantitative analysis of BoNT serotype A1 (BoNT/A1), the most common toxin serotype. The method can detect as little as 400 fg/mL of solid powder toxin resulting in a broad quantitation range from 0.4–100 pg/mL and can be accomplished in approximately an hour. Additionally, to show that it would be useful for clinical samples during a potential exposure, the method was applied to the analysis of spiked human serum and urine samples. The limits of detection (LODs) are 200 fg/mL for serum (in 25% dilution) and 1.00 pg/mL for urine (in 10% dilution), with sample quantitation ranges from 0.8–400 pg/mL and 10–1000 pg/mL, respectively.

Keywords Botulinum neurotoxin, Immunoassay, Single-molecule detection, Single-molecule array method, Human serum, Human urine

Introduction

BoNT exists in seven distinct known serotypes, A through G, many having multiple subtypes, which are all produced by spore-forming *Clostridium botulinum* [1]. The most common route of intoxication is through oral ingestion of preformed toxin in contaminated food (typically home-canned food) leading to food-borne botulism. Due to its extreme potency and relative ease of production and distribution, BoNT poses a threat to the public as a bioweapon. The lethal doses of BoNT serotype A in human are approximately 1 ng/kg intravenously or intramuscularly and about 1 µg/kg orally [1]. Therefore, BoNTs are classified as CDC Category A (highest risk) bioterrorism agents. Regardless of the causative toxin type, human BoNT intoxication displays similar clinical signs including difficulty in seeing, speaking, and/or swallowing. At advanced stages, further complications of intoxication can lead to loss of head control, hypotonia (low muscle tone), and myalgia (general muscle weakness) [1]. Lethality generally results from respiratory failure, and severely exposed patients must be maintained for up to months on a ventilator. Botulism and BoNT toxicity are frequently misdiagnosed as diseases that have similar clinical symptoms, including Miller-Fisher syndrome, myasthenia gravis, or a disease of the central nervous system [1]. For example, an unintentional food contamination in Canada during the late summer of 1985 resulted in one of the largest and most infamous botulism outbreaks. All 28 persons, who became ill within a 6-week period, were misdiagnosed [2]. It is therefore critical to have a rapid, accessible, and sensitive diagnostic method for timely recognition of a botulism outbreak or an intentional release, to prevent further cases of misdiagnosis and the resulting complications.

The current standard test for BoNT is the mouse bioassay, which can detect this toxin at a LOD of approximately 20 pg/mL [3]. However, this test requires from 2–8 days for the results and is currently available only at the CDC and at around 20 state health laboratories. It also requires a cell culture assay to determine the serotype of the toxin [1]. The mouse bioassay is therefore highly time-consuming and not readily available for timely diagnosis of botulism or BoNT poisoning. In cases of BoNT intoxication, antitoxin is effective in reducing the severity of symptoms if administered early—ideally less than 24 h after the onset of the symptoms [4]. Other laboratory methods for detecting BoNT include enzyme-linked immunoassay (ELISA) and immuno-polymerase chain reaction (immuno-PCR) that have LODs in the range of 60 pg/mL. However, these tests are highly labor-intensive and can take up to 5–8 h for results [3]. In addition, PCR is only designed to detect the presence of bacterial spores or the botulinum

neurotoxin genes; thus it would not be useful if exposure occurred with the purified toxin. To address these problems, we tested the Simoa method for quantitative analysis of BoNT in white powder and in human biological matrices (serum and urine), employing high affinity single domain antibody detection agents called VHHs.

Materials and methods

Simoa homebrew assay kit was purchased from Quanterix Inc. (Lexington, MA, USA) that included carboxyl-functionalized paramagnetic beads (2.7-μm in diameter), EZ-Link NHS-PEG4 biotin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), streptavidin-β-galactosidase (SβG) enzyme concentrate, resorufin-β-D-galactopyranoside (RDG) substrate, diluents (bead diluent, detector diluent, and SβG diluent), and buffers (bead conjugation buffer, bead wash buffer, bead blocking buffer, biotinylated reaction buffer, wash buffer 1, and wash buffer 2); BoNT/A1 from Metabiologics Inc. (Madison, WI, USA). Recombinant camelid anti-BoNT/A1 heavy-chain-only Ab V_H (VHH) binding domains were made in the Shoemaker Lab (Tufts Cummings School of Veterinary Medicine, Grafton, MA, USA) [5]; Amicon Ultra-0.5 mL centrifugal filters 3kD from EMD Millipore (Billerica, MA, USA); Simoa HD-1 Analyzer from Quanterix Inc. (Lexington, MA, USA); unidentified human serum and urine samples from healthy subjects from BioreclamationIVT (Chestertown, MD, USA).

Preparation of capture beads with VHH antibodies

One hundred microliters of 2.3 x 10^9/mL carboxyl-functionalized paramagnetic beads (total 2.3 x 10^8 beads) were washed three times with bead wash buffer and two times with bead conjugation buffer. The washed beads were suspended in 190 μL of bead conjugation buffer, followed by the 10-μL addition of 10 mg/mL of EDC in bead conjugation buffer solution. The total 200 μL mixture was incubated while shaking for 30 min, followed by one time washing with bead wash buffer. The washed beads were incubated with 200 μL of 0.5 mg/ml VHH antibody solution while shaking for 2 h. Then, beads were washed two times with bead wash buffer, followed by the addition of 200 μL of bead blocking buffer. The mixture was incubated while shaking for 30 min. Finally, beads were washed once with bead wash buffer, once with bead storage buffer, and were stored at 4°C in 200 μL of bead storage buffer.

Biotin conjugation of VHH antibodies

The Amicon 3kD mwco spin column was used to exchange the VHH's storage buffer to biotinylation reaction buffer. A 100-µg aliquot of VHH in storage buffer was added to the spin column. The column was filled up to the 500-µL mark using biotinylation reaction buffer, and was centrifuged at 14,000 x *g* for 30 min. The supernatant was discarded and the column was centrifuged once more at 14,000 x *g* for 30 min with 500 µL of biotinylation reaction buffer. The supernatant was discarded and the column was transferred to a new Amicon tube, where the VHH was recovered in biotinylation reaction buffer by inverting and centrifuging the column at 14,000 x *g* for 30 min. Then, 3 µL of 3.4 mM of EZ-Link NHS-PEG4 biotin was added to 100 µL of 1 mg/mL VHH antibody in biotinylation reaction buffer. The total 103 µL mixture was incubated at room temperature without shaking for 30 min. The reaction was stopped and the biotin-VHH antibody was purified by using the Amicon 3kD mwco spin column.

Capture of BoNT/A1 and formation of enzyme-labeled immunocomplexes

Solutions of 5 x 10^6/mL antibody coated paramagnetic beads, 0.03 µg/mL biotin-detection antibody, and 150 pM SβG solution were separately loaded into 14-mL plastic bottles. The 100 µM RDG solution was loaded into a 4-mL plastic bottle. The bottles were loaded onto Simoa HD-1 Analyzer for automatic dispensing. Ten-fold dilutions of BoNT/A1, 0.01–1000 pg/mL, were prepared in triplicate in 1X phosphate buffered saline (PBS) containing 0.3% Tween 20 (T20) and 0.1% bovine serum albumin (BSA) on a 96-well plate. 500,000 antibody captured beads were incubated with 100 µL of each BoNT/A1 concentration for 15 min. Captured beads were then washed and were incubated with 100 µL of biotin-detection antibody solution for 5 min. The beads were then washed and were incubated with 100 µL of SβG solution for 5 min. A step-by-step schematic procedure of BoNT/A1 Simoa assay is shown in Fig. 1. All incubations were performed while shaking.

Imaging and Analysis

Enzyme-labeled immunocomplexed beads were suspended in 25 µL of RDG solution. Then, 15 µL of well-suspended captured beads in RDG solution were loaded by gravity onto the 216,000 femtoliter-reaction wells on a single array of a 24-array Simoa disc[6]. The wells were sealed with fluorocarbon oil [7]. "On" wells containing the enzyme-labeled immunocomplexed beads will turn over the RDG substrate to generate resorufin and are imaged using a built-in charge-coupled device camera and standard imaging optics within the Simoa HD-1 Analyzer[8]. Using 46-fL sized reaction wells, a high local concentration of fluorescent product is formed in the "on" wells, which lead to the ultra-sensitive detection capability of Simoa. By counting the number of "on" wells compared to the total number of the beads loaded into the wells, we obtain a digital readout of the protein concentration in the original samples. At higher concentrations of the target protein or in analog range, when each bead is labeled with at least one immunocomplex, the average fluorescence intensity of the "on" wells is used to determine the concentration of the target protein. The average enzyme per bead (AEB) is used as the standard unit of measurement for both the digital and analog ranges in a Simoa assay.

Results and discussion

Serial dilutions of BoNT/A1 powder toxin in 1X PBS buffer (0.3% T20, 0.1% BSA) ranging between 0.01–100 pg/mL (n=3) were analyzed using the Simoa method. Control samples (n=3) were prepared without the toxin. As little as 400 fg/mL, or 40 fg in a 100-µL sample, was detectable using the present method (Fig. 2 and Table 1). Resulting data were fitted using a four parameter logistic curve that had $R^2 > 0.99$. The LOD was determined by extrapolating the concentration from the background plus three times the standard deviation of the background. This method is therefore applicable to detect suspected powder toxin samples with a broad and ultrasensitive quantitation range of 0.4–100 pg/mL. In an average 70-kg adult, the lethal doses are 100 ng intravenously or intramuscularly, and 70 µg orally. Therefore, considering that each Simoa assay uses 100 µL of sample, the Simoa assay's sensitivity is at least 2.5×10^6 times below the intravenous lethal dose, at which there would not be any clinical symptoms. In addition, the Simoa can be applied in situations where there is only a trace amount of toxin. Even at 1000X dilution, the samples that have ~40 pg or more (2.5×10^3 times below the lethal dose) are detectable by using the present Simoa method. The assay was accomplished in 64 min and the results were robust and reproducible at all concentrations (CV~3–23 %).

As mentioned earlier, we are interested in applying the Simoa method to the detection of BoNT/A1 in human samples. This application is essential in cases where patients are admitted with suspected BoNT poisoning but the original toxin sample is not available for testing. A serum sample of the patient can be drawn and subsequently tested for BoNT/A1 using the present Simoa method. It is estimated that a 70-kg adult has approximately 5 liters of blood. Assuming the injected toxin is evenly distributed throughout the entire 5 liters, in order to detect the trace amount of toxin in a small sample of serum of ~100 µL (50,000 times diluted), an ultrasensitive method is needed. We applied Simoa for the detection of BoNT/A1 in spiked serum samples ranging between 0.01–100 pg/mL at 25%, 10% and 1% serum dilution in 1X PBS buffer (0.3% T20, 0.1% BSA) (Table 1). The lowest background and highest signal-to-noise ratios (S/N) were achieved with 25% serum with a LOD of 200 fg/mL (Fig. 3). Using the 4-fold dilution in serum extended the quantitative range of BoNT/A1 Simoa assay from 0.8–400 pg/mL and reduced the required sample volume to 25 µL. With a serum sensitivity of 0.8 pg/mL, any intravenously or injected amounts greater than 4 ng in total, 25 times less than the lethal dose, are detectable using this method. These results demonstrate that the Simoa method is highly applicable to the quantitative analysis of BoNT/A1 in human serum samples.

In addition, we used Simoa assay to detect BoNT/A1 in urine, to test for any possible matrix interference. Similarly, BoNT/A1 was spiked in human urine ranging between 0.01–100 pg/mL at 25%, 10% and 1% urine dilution in 1X PBS buffer (0.3% T20, 0.1% BSA) (Table 1). We obtained a LOD of 1.00 pg/mL with the lowest background and highest S/N ratios at 10% urine dilution, resulting in a sample quantitative range of 10-1000 pg/mL (Fig. 3). Thus, there are insignificant interferences when using urine as sample matrix for toxin detection.

Most of the reported detection methods for BoNT focus on the detection of toxin in food and water to identify foodborne botulism. However, the increasing concern for BoNT as a biological weapon requires a reliable method for toxin detection in clinical matrices such as human serum. The detection in clinical matrices remains difficult due to toxin sequestration within the body making the amount of detectable toxin in biological samples much lower than the exposure amount [1]. Therefore, the detection method should be as sensitive as possible to maximize the practical utility of the assay. In this study, we demonstrated the ultrasensitive detection of BoNT/A1 using the Simoa method that is applicable to both powder toxin incidents (LOD at 400 fg/mL) and human biological matrices (LODs at 200 fg/mL and 1.00 pg/mL for serum and urine, respectively). Our Simoa assay provides a rapid, simple, and

ultrasensitive detection method in human biological matrices to provide supportive care and address public health emergencies.

Conclusions

The Simoa assay described here has demonstrated the ultrasensitive detection of BoNT/A1. The assay is significantly more sensitive and faster than mentioned methods. The enhanced performance of the Simoa assay provides clinically actionable information in a timeframe where there is only a short window for intervention. For future work, the Simoa assay can be developed for other serotypes of BoNT, ideally with detection agents recognizing all of the serotypes. A multiplexed assay that is capable of specifically detecting one or all other serotypes would be ideal for biothreat applications and can be adapted using the previously reported multiplexed format of Simoa for up to 6-plex detection [9].

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Conflict of interest The authors declare the following competing financial interest(s): David R. Walt is the scientific founder and a board member of Quanterix, Corp. All remaining contributing authors declare no competing financial interests.

Ethical approval Research does not involve human participants and/or animals. Unidentified human serum and urine samples were purchased commercially. No informed consent was needed by researchers, according to the guidelines of National Institutes of Health (NIH).

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Figures and Table

Table 1 Effects of various matrices on the responses of the Simoa detection assay to BoNT/A1

Matrix	Dilution factor	Coefficient of determination (R^2)	Limit of detection (pg/mL)	Quantitation range (pg/mL)	Coefficient of variation (%)
PBS buffer		0.998	0.4	0.4 – 100	3.22 – 22.8
Serum	25%	1.000	0.2	0.8 – 400	3.95 – 21.5
	10%	1.000	0.3	3 – 3000	3.44 – 14.51
	1%	0.996	0.3	30 – 10000	2.03 – 38.5
Urine	25%	1.000	2.0	8 – 400	0.51 – 39.6
	10%	0.998	1.0	10 – 1000	1.59 – 25.3
	1%	0.990	0.6	60 – 10000	1.43 – 67.9

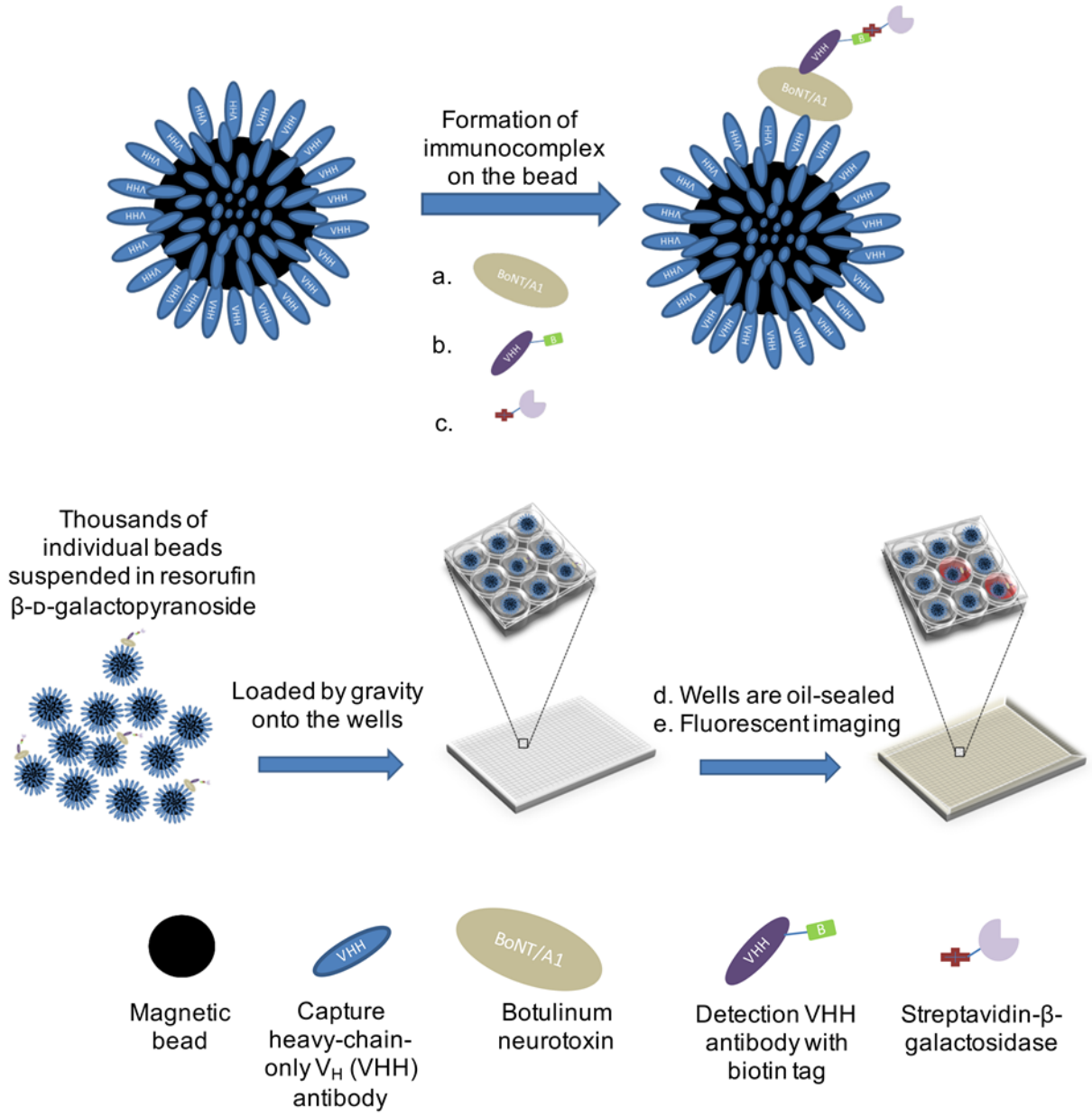

Fig. 1 Schematic procedure of botulinum neurotoxin serotype A1 detection using the single-molecule array (Simoa) method. VHH antibody–labeled beads (**a**) are used to capture BoNT/A1 in the sample, and subsequently (**b**) detected by a secondary biotin-conjugated VHH antibody (**c**) labeled with SβG enzyme, forming an enzyme-labeled immunocomplex on the beads. Thousands of individual beads, with or without the immunocomplexes, are suspended in RDG substrate and loaded by gravity onto the femtoliter wells. The wells are (**d**) isolated from each other by fluorocarbon oil sealing, and (**e**) the fluorescent images are taken for the entire well array to determine the "on" and "off" wells

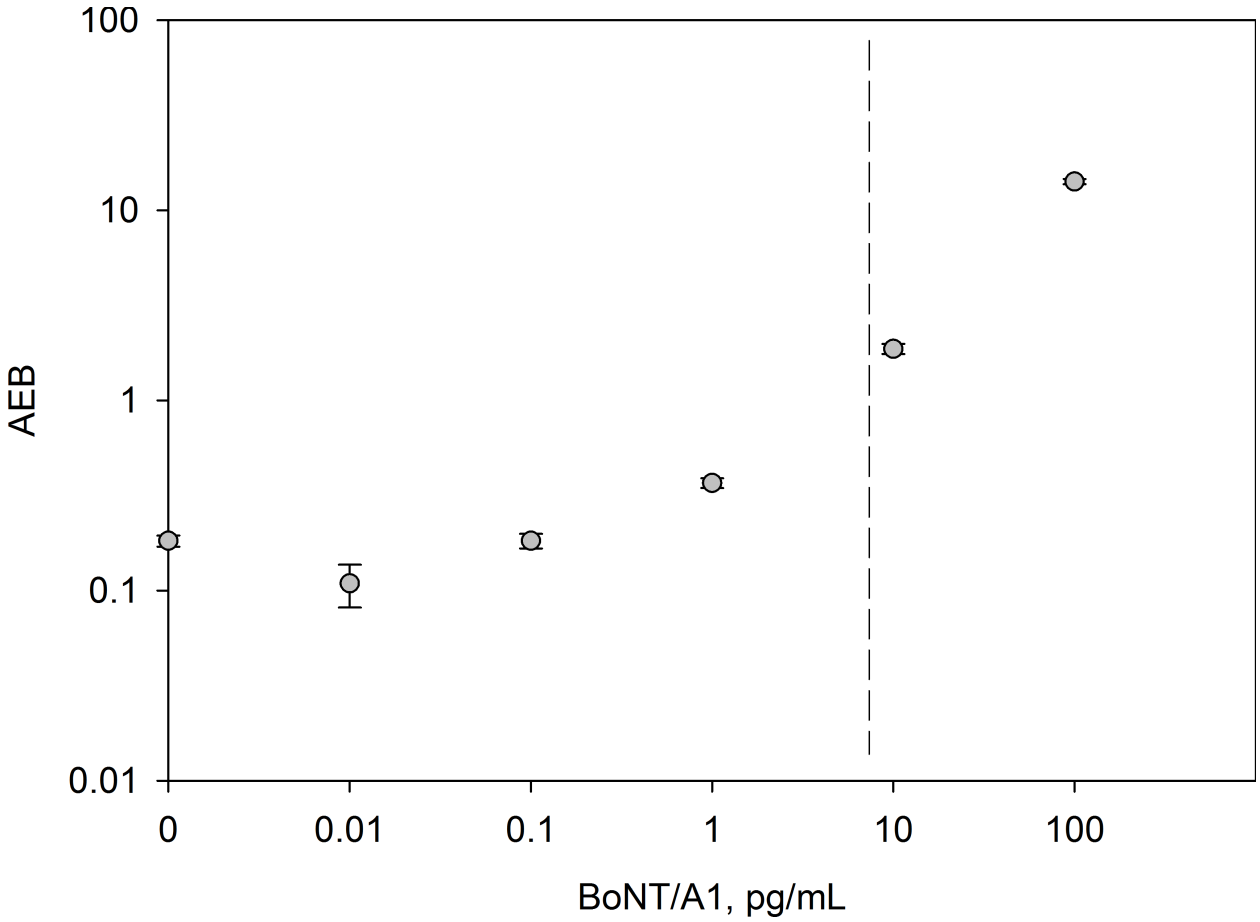

Fig. 2 Detection of powder BoNT/A1 in 1X PBS buffer using the Simoa method. The log-log standard curve, R^2>0.99, provided a calculated LOD of 400 fg/mL. The dashed line indicates the boundary between the digital and analog readouts. Experiments were performed in triplicate and the error bars represent the standard deviations. *AEB* is a measure of the number of average enzyme molecules per bead.

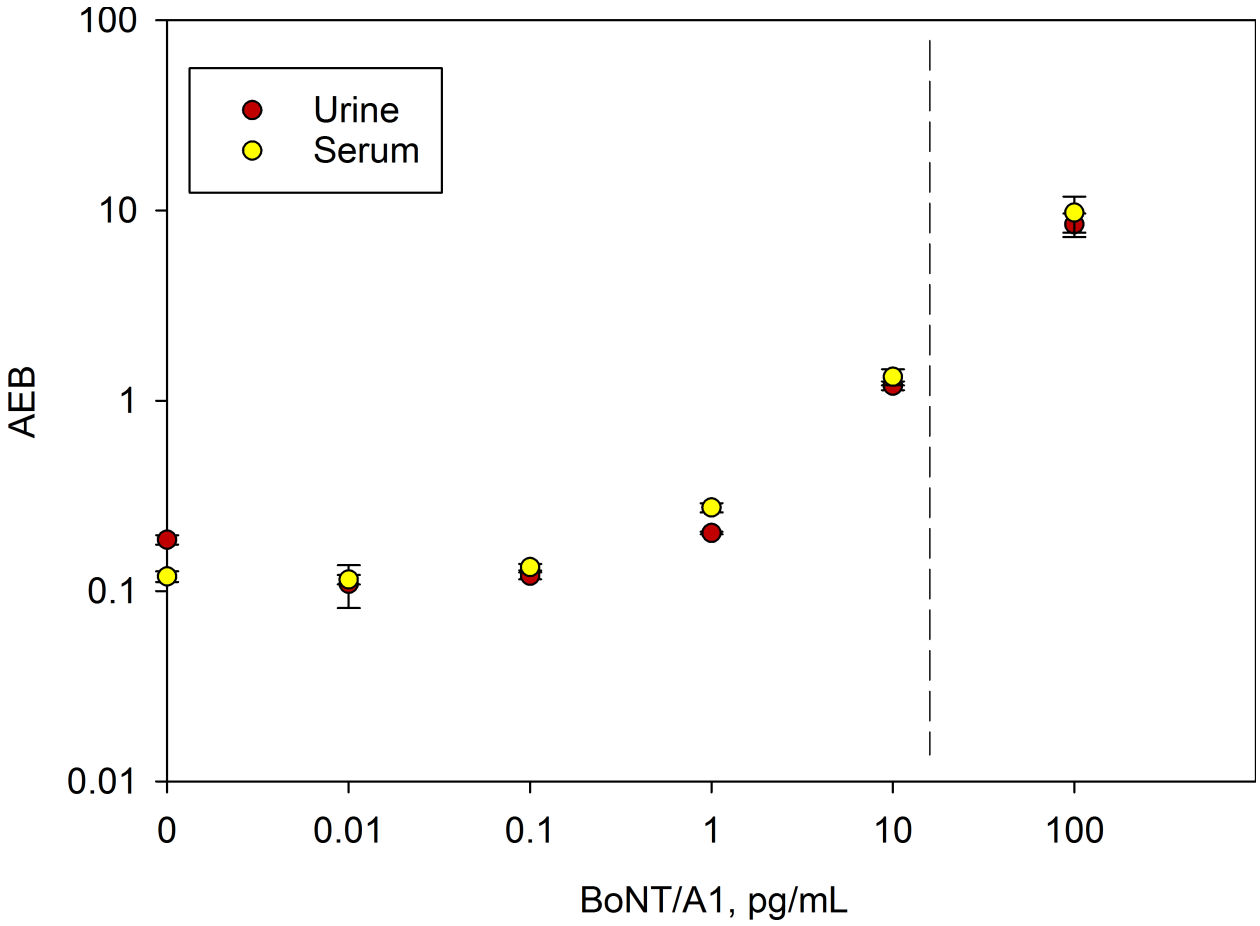

Fig. 3 Detection of BoNT/A1 in spiked human serum and urine samples using the Simoa method. The log-log standard curves in each matrix, R^2>0.99 for both, provided calculated LODs of 200 fg/mL and 1.0 pg/mL for serum (yellow) and urine (red), respectively. The dashed line indicates the boundary between the digital and analog readouts. Experiments were performed in triplicate and the error bars represent the standard deviations.