DESIGN OF A TWO-PLEX ASSAY FOR DETECTION OF *CLOSTRIDIUM DIFFICILE* TOXINS A AND B

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INTRODUCTION

Clostridium difficile infection (CDI) is an inflammation of the large intestine due to an infection with a spore-forming bacteria, *C. difficile*, causing diarrhea. It is a healthcare-associated disease linked to the use of antibiotics. Infection with *C. difficile* species is common, serious, and costly.

The presence of *C. difficile* toxin in fecal samples is the most reliable indicator of true CDI, but immunoassays for toxin testing are not suitable as stand-alone tests due to a lack of sensitivity. All guidelines strongly recommend a two-step assay algorithm based on the detection of bacteria followed by toxin detection.

Since 2010, many laboratories are performing molecular assays for toxin gene detection and an increase in CDI incidence has been observed. Nevertheless, the presence of genes does not always correlate with the presence of functional toxins, leading to an inability to distinguish a disease state from colonization.

An automated 1-step assay with high sensitivity for *C. difficile* toxins could strongly improve the accuracy of CDI diagnosis and reduce costs.

OBJECTIVE

The objective of this study was to evaluate a quantitative assay using the Simoa™ technology for simultaneous detection of toxins A and B of *C. difficile* in human fecal samples.

PRINCIPLE OF SIMOA TECHNOLOGY

The Simoa technology is based upon the isolation of individual paramagnetic bead in wells, allowing for a "digital" readout of each bead to determine if it is bound to the target analyte or not.

The assay consists of a standard ELISA conducted with paramagnetic microbeads, followed by the isolation of individual beads in microwells of an array for digital imaging.

The analyte is first captured by an antibody (Ab) coated on paramagnetic microbeads, and then detected by an antibody directly conjugated β-galactosidase. The simultaneous detection of both toxins is possible thanks to the use of encoded (or fluorescent) beads and of two detectors specific to each toxin.

After the final wash, the beads are re-suspended in a buffer containing the substrate, resorufin β-D-galactopyranoside, and transferred into an array containing 216 000 wells fitted to hold no more than one bead per well.

Each fluorescent bead type are detected by the Simoa imaging module as well as the detection of bead-associated enzymatic activity by digital analysis (presence or absence of immune complex in well) and by analog measure (measure of fluorescence intensity in well).

The whole signal range is determined using imaging analysis software to obtain an Average Enzyme per Bead (AEB).

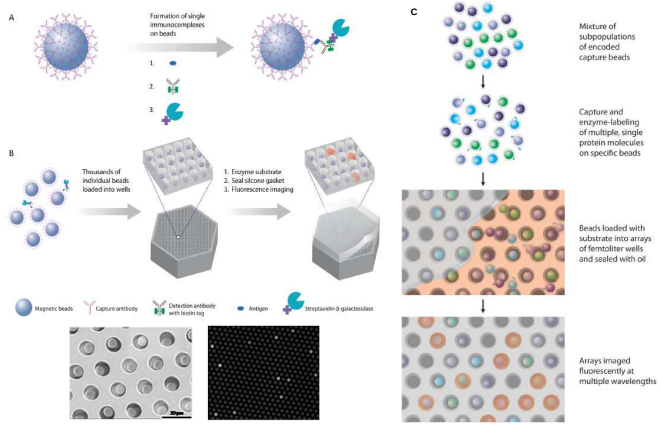

Figure 1: Simoa Technology
A: standard ELISA; **B:** bead isolation for digital analysis; **C:** Multiplex technology

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RESULTS

The specificity of the antibodies of each assay was evaluated. We observed that each Ab pair was specific to toxin A and B, respectively (Figure 2).

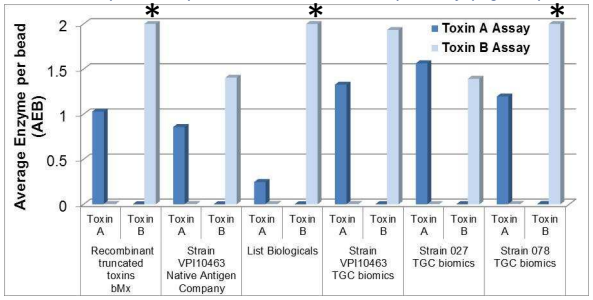

Figure 2: Antibody Specificity for each assay
Toxins tested at 1000 pg/ml ; ✱: signal above 2 AEB

The interference of toxin A on the accuracy of toxin B assay, and inversely was also evaluated (Figure 3). No impact of high concentrations of toxin A (up to 100 ng/ml) on the accuracy of toxin B assay was observed, while concentrations of toxin B superior or equal to 10 ng/ml interfered with the accuracy of toxin A assay, specially at low concentrations (0-10 pg/ml). This interference was observed with three different Ab pairs.

Difference in AEB values obtained are observed with toxins from Native Antigen Company (Oxfordshire, U.K.) and TGC biomics (Bingen, Germany). It can be linked to the methods used for toxin concentration determination (absorption at 280nm versus ELISA for the determination of toxin concentration).

Even if there is a slight impact in the accuracy of toxin A concentration when samples contain high concentration of toxin B, it will not impact the clinical decision regarding C. diff diagnosis.

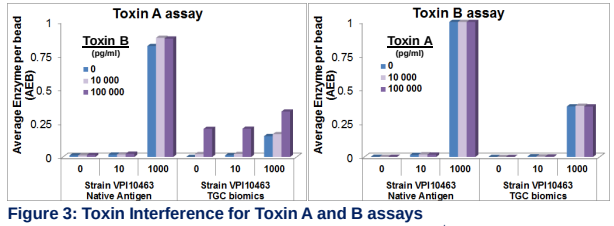

Figure 3: Toxin Interference for Toxin A and B assays
Toxins tested at 0, 10 or 1 000 pg/ml alone or combined with the 2nd toxin up to 100 ng/ml

To evaluate the multiplex capability of the Simoa technology, several dye-encoded beads from Quanterix (MA, USA) were tested and compared to non-encoded beads: similar results were obtained in term of dynamic range as shown on Figure 4 with beads labeled /encoded with a 488 fluorophore and non-encoded beads. Furthermore, the dynamic range obtained for both assay evaluated in a monoplex and in a duplex format showed also similar results (Figure 5). The estimation of limit of detection for the native toxin A was < 0,5 pg/ml in duplex and monoplex format, and for the native toxin B, < 2 pg/ml.

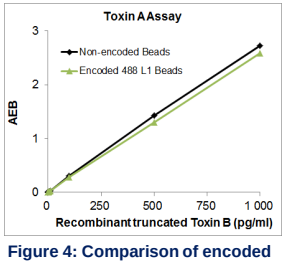

Figure 4: Comparison of encoded and non-encoded beads

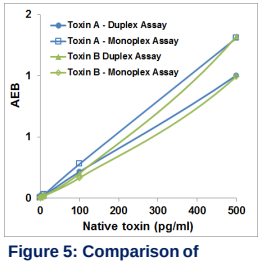

Figure 5: Comparison of monoplex assay and duplex assay

The cross-reactivity of the Abs was then evaluated in the duplex format. We observed that anti-toxin B Ab used as capture on beads was specific to toxin B, while anti-toxin A Ab as capture could detect small quantities of toxins B when anti-toxin B Ab was used as detector (Figure 6).

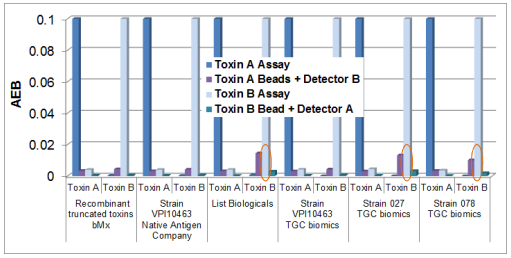

Figure 6: Antibody Specificity in duplex format
Toxins tested at 1000 pg/ml

Finally, we evaluated the impact of one toxin on the other assay in a duplex format. A non specific signal was observed in the duplex assay when the response for one analyte was high.

Nevertheless, the increase in background was low, few (< 3-5 pg/ml) of one toxin was detected when sample contained the other toxin at a concentration of 1 000 pg/ml (Table 1).

It was not be due to reagent specificity as we did not observe interference in monoplex assay at this concentration of native toxin. It seems that it was due in part to an optical phenomena called spatial crosstalk.

Native Toxins (1000 pg/ml)	Monoplex Assay				Duplex Assay	
	Toxin A Assay	Beads Toxin A Detector Toxin B	Toxin B Assay	Beads Toxin B Detector Toxin A	Toxin A Response	Toxin B Response
Native A	3.0913	0.0003	0.0002	0.0017	3.7605	0.0074
Native B	0.0024	0.0003	0.1172	0.0019	0.0042	0.1201
Diluent	0.0019	0.0001	0.0002	0.0019	0.0018	0.0018

Table 1: Increase in background in duplex format
Toxins from Native Antigen Company

CONCLUSION

The Simoa technology allow to develop multiplex assay by using beads encoded with specific fluorophore. **Similar performance have been obtained for monoplex assay and duplex assay on the Simoa instrument with non-encoded and encoded beads.**

The **antibodies pairs selected for toxin A and toxin B assays were specific to each toxin, respectively, in monoplex format**. Nevertheless, we observed with a toxin B from a specific supplier an impact in toxin A assay accuracy when there is very high concentrations (≥ 100 ng/ml) of this toxin B in sample. Furthermore, we showed that **anti-toxin A Ab as capture could detect small quantities of toxins B when anti-toxin B Ab was used as detector**. These results are not very surprising knowing that there is a homology of sequence between toxins A and B of 63% amino acid. Finally, we observed an increase in signal for both assay when the concentration of the other analyte is high (1 ng/ml) which is due in part to an optical phenomena of the technology.

In conclusion, **it is possible to develop a duplex assay for the simultaneous detection of both toxins using the Simoa technology**. However the measuring range of the toxin A assay may be more restricted in a duplex than in monoplex due to the low cross-reactivity of the toxin A Abs and the optical phenomena. For the next steps, **the importance of each toxin, A and B, in diagnosis and pathology will be first defined/assessed by using the two assays separately**.