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Laboratory comparison between cell cytotoxicity neutralization assay and ultrasensitive single molecule counting technology for detection of *Clostridioides difficile* toxins A and B, PCR, enzyme immunoassays, and multistep algorithms

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ABSTRACT

Diagnostic tests for *Clostridioides difficile* infection (CDI) lack either specificity (nucleic acid amplification tests) or sensitivity (enzyme immunoassays; EIAs). The performance of the Singulex Clarity® C. diff toxins A/B assay was compared to cell cytotoxicity neutralization assay. Testing was also performed using an EIA for glutamate dehydrogenase (GDH) and *C. difficile* toxins A and B (C. Diff Quik Chek Complete®), polymerase chain reaction (PCR) (BD MAX™ Cdiff Assay), and 2 multistep algorithms: algorithm 1 (discordant GDH/toxin results arbitrated by PCR) and algorithm 2 (PCR-positive samples tested with toxin EIA). The Clarity assay and PCR both had 97% sensitivity, while specificity was 100% for Clarity and 79% for PCR. Algorithm 1 yielded 41% discordant results, and both toxin EIA and algorithm 2 had 58% sensitivity. Median toxin concentrations, as measured by the Clarity *C. difficile* toxin assay, were 3590, 11.5, 0.4, and 0 pg/mL for GDH+/toxin+, GDH+/toxin−/PCR+, GDH+/toxin−/PCR−, and GDH−/toxin− samples, respectively ($P < 0.001$). The Clarity assay may offer a single-test solution for CDI.

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1. Introduction

Clostridioides (formerly *Clostridium*) *difficile* is highly contagious and can lead to *C. difficile* infection (CDI) with severe complications and death (Burnham and Carroll, 2013; Lessa et al., 2015). A patient diagnosed with CDI is subject to infection-control measures, including contact isolation, and treatment with antibiotics (McDonald et al., 2018; Surawicz et al., 2013). CDI is a clinical diagnosis, requiring the presence of acute diarrhea and identification of either *C. difficile* toxins A (TcdA) and/or B (TcdB) or toxigenic species in stool (McDonald et al., 2018). Individuals can be colonized asymptomatically with toxigenic *C. difficile*, but colonization does not always lead to CDI and does not, by itself, justify treatment (Burnham and Carroll, 2013; Fang et al., 2017; Planche et al., 2013; Polage et al., 2015). Isolation of patients is costly, and CDI treatment can itself make the patient more vulnerable to the disease (Fang et al., 2017). Developing a diagnostic test for rapid and sensitive detection of the toxins associated with disease is a high priority, along with the identification and development of biomarkers specific for CDI (Crobach et al., 2016; McDonald et al., 2018; Surawicz et al., 2013).

Identification of toxigenic strains can be accomplished by using either toxigenic culture or nucleic acid amplification test (NAATs), e.g., polymerase chain reaction (PCR). Toxigenic culture is labor intensive and has a long turnaround time. NAATs, while costly, are rapid and highly sensitive. However, as NAATs only detect *C. difficile* toxin gene(s), not the free toxins, their clinical specificity and correlation with infection or outcome are debatable (Burnham and Carroll, 2013; Fang et al., 2017; Planche et al., 2013; Polage et al., 2015).

The reference method for detection of free toxins in stool is the cell cytotoxicity neutralization assay (CCNA). The turnaround time of CCNA is up to 48 h, which is not appropriate to adequately address the clinical need for rapid diagnosis and infection control. Currently available enzyme immunoassays (EIAs) for the detection of toxins are rapid and specific, but they are limited by moderate sensitivity. EIAs for detection of *C. difficile*-specific glutamate dehydrogenase (GDH) are rapid and highly sensitive but have low clinical specificity

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(Burnham and Carroll, 2013; Pollock, 2016). Consequently, since all current methods have shortcomings in 1 or more areas as explained above, current guidelines recommend various multistep testing algorithms for the diagnosis of CDI, such as GDH-and-toxin EIA arbitrated by NAAT, or NAAT plus toxin, or NAAT alone if preagreed institutional criteria for patient stool submission are met (Crobach et al., 2016; McDonald et al., 2018; Surawicz et al., 2013).

The ultrasensitive Singulex Clarity® C. diff toxins A/B assay (Clarity), in development for the Singulex Clarity system at the time of the study, is an ultrasensitive immunoassay for the detection of TcdA and TcdB in stool. The Clarity system is an automated, in vitro diagnostic platform powered by Single Molecule Counting technology that utilizes a scanning confocal optical system for analyte detection.

In this study, the laboratory performance of the Clarity assay relative to CCNA was determined. The performance was compared with PCR, GDH-and-toxin EIA, and multistep algorithms.

2. Materials and methods

2.1. Singulex Clarity C. diff toxins A/B assay

The Singulex Clarity C. diff toxins A/B assay (in development at the time of the study), which detects TcdA and TcdB in stool on the Singulex Clarity system, has been described previously (Sandlund et al., 2018). Briefly, either 100 μL of liquid or semisolid or 0.1 g of a solid stool sample is mixed (1:20) with diluent buffer (Tris-buffered saline–EDTA with 3% bovine serum albumin) and centrifuged at 14,000 ×*g* for 10 min, and 300 μL of the resulting supernatant is loaded onto the Singulex Clarity system. Once on board, the sample is mixed with paramagnetic microparticles precoated with anti-TcdA and anti-TcdB monoclonal antibodies (capture reagent) and fluorescently labeled toxin-specific antibodies (detection reagent), and incubated at 37 °C for 5 min in a reaction vessel. After incubation, unbound material is washed away, and an elution buffer is added to dissociate the immune complexes from the paramagnetic microparticles. The resulting mixture is exposed to a magnetic field to separate the paramagnetic microparticles from the dissociated fluorescently labeled antibodies, and the resulting eluate is transferred to a detection vessel where the dye-labeled molecules are detected. A proprietary algorithm counts detected events and compares these to a previously established standard curve. The Singulex Clarity software interpolates the data into a combined TcdA/TcdB concentration. The limits of detection for TcdA and TcdB are 0.8 and 0.3 pg/mL in buffer and 2.0 and 0.7 pg/mL in stool, respectively (Sandlund et al., 2018). The total assay turnaround time is 32 min.

2.2. Cutoff establishment

A detailed description of the cutoff establishment is provided elsewhere (Sandlund et al., 2018). In short, 103 frozen, deidentified stool samples from patients with suspected CDI (not all-comers), provided by TriCore Reference Laboratories (Albuquerque, NM) and Discovery Life Sciences Laboratories (Los Osos, CA), were used to determine a preliminary cutoff for the Clarity assay using CCNA as the reference method. The samples, 27 CCNA+ and 76 CCNA−, were tested in triplicate. The toxin concentration that minimized the difference between sensitivity and specificity was used to set the cutoff (Habibzadeh et al., 2016), and the diagnostic performance characteristics (sensitivity and specificity) were determined.

2.3. Clinical performance

Ninety-five residual deidentified stool samples from patients with suspected CDI were acquired from Tricore Reference Laboratories. A research technician assigned a Bristol scale value to each residual sample (79 Bristol scales 5–7, 16 Bristol scale 4). A Bristol scale assessment on samples for CDI testing was not put in place at the time of the study. Forty-three (45.3%) of the patients were women, and 92 (96.8%) were age 18 years or older. Samples were stored at 2–8 °C and tested <72 h after collection with the Clarity assay, a rapid EIA for the detection of GDH, TcdA, and TcdB (C. Diff Quik Chek Complete®, TechLab, Inc., Blacksburg, VA), and PCR for the detection of *tcdB* (BD MAX™ Cdiff Assay, Becton, Dickinson and Company, Franklin Lakes, NJ). The C. Diff Quik Chek Complete test detects levels of toxin A at ≥630 pg/mL, toxin B at ≥160 pg/mL, and GDH at ≥800 pg/mL (Package Insert C, n.d.). The performance of each test was evaluated against CCNA (*C. difficile* TOX-B Test®, TechLab; tested at ARUP Laboratories, Salt Lake City, UT) and compared with 2 multistep algorithms: EIA testing with discordant GDH-and-toxin results arbitrated by PCR (algorithm 1) and PCR testing with positive samples tested with toxin EIA (algorithm 2).

2.4. Statistical methods

The Kruskal–Wallis test was used to compare toxin concentrations across more than 2 unordered groups, and analysis of variance contrast models were used to test for significant trends across more than 2 ordered groups. Statistical analysis was performed with SAS v9.4, Analyze-It for MS Excel 4.51, and GraphPad Prism version 7 software packages.

3. Results

3.1. Cutoff establishment

A preliminary cutoff for the Clarity assay was set at 16.7 pg/mL (Fig. 1). The area under the receiver operating characteristic (ROC) curve yielded a *C*-statistic of 0.99 with a sensitivity at 96.3% and a specificity at 96.1% compared to CCNA.

3.2. Performance characteristics

In this sample set, there were 33 CCNA+ and 62 CCNA− samples. Compared to CCNA, the Clarity assay demonstrated 97.0% sensitivity and 100% specificity, while PCR alone had 97.0% sensitivity and 79.0% specificity. Toxin EIA had 57.6% sensitivity and 100% specificity. GDH-

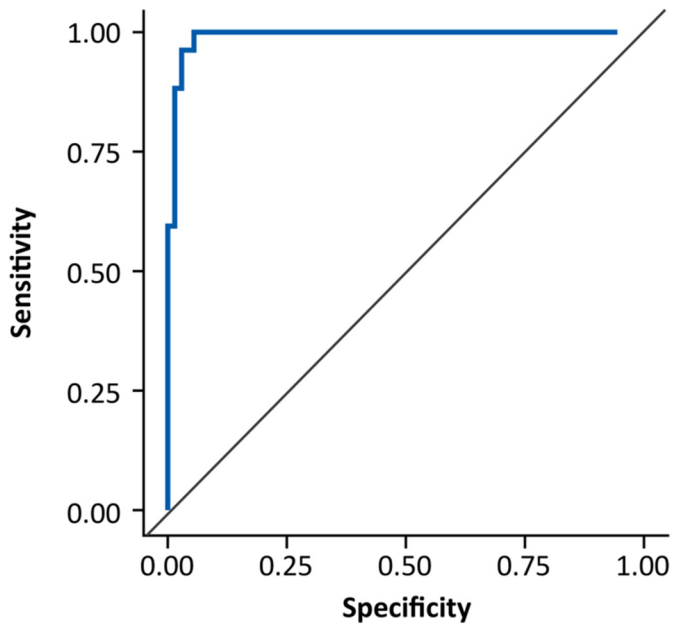

Fig. 1. A preliminary cutoff for the Singulex Clarity C. diff toxins A/B assay was set at 16.7 pg/mL. The areas under the ROC curve demonstrated a *C*-statistic of 0.99 (95% CI 0.98–1.00) with a sensitivity at 96.3% (95% CI 81.0–99.9) and a specificity at 96.1% (95% CI 88.9–99.2) compared to CCNA.

Table 1
Performance of the Singulex Clarity C. diff toxins A/B assay, PCR (BD MAX Cdiff Assay), toxin EIA (C. Diff Quik Chek Complete), GDH-and-toxin EIA (C. Diff Quik Chek Complete), and multistep algorithms compared to cell cytotoxicity neutralization assay. Algorithm 1: discordant GDH-and-toxin EIA results arbitrated by PCR. Algorithm 2: PCR+ samples tested with toxin EIA. CCNA was utilized as the reference test.

	Samples tested ($n = 95$)				
	CCNA+ ($n = 33$)		CCNA- ($n = 62$)		
Testing method	*Test positive (% Sensitivity)*	*95% CI*	*Test negative (% specificity)*	*95% CI*	*Discordant results (%)*
Singulex Clarity C. diff toxins A/B assay	32/33 (97.0)	(82.5–99.8)	62/62 (100)	(92.7–100)	N/A
PCR	32/33 (97.0)	(82.5–99.8)	49/62 (79.0)	(66.4–87.9)	N/A
Toxin EIA	19/33 (57.6)	(39.4–74.0)	62/62 (100)	(92.7–100)	N/A
GDH-and-toxin EIA	19/33 (57.6)	(39.4–74.0)	37/62 (59.7)	(46.4–74.7)	39 (41.1%) GDH+/toxin−
Algorithm 1	32/33 (97.0)	(82.5–99.8)	49/62 (79.0)	(66.4–87.9)	N/A
Algorithm 2	19/33 (57.6)	(39.4–74.0)	62/62 (100)	(92.7–100)	26 (27.4%) PCR+/toxin−

Abbreviations used: CCNA = cell cytotoxicity neutralization assay; PCR = polymerase chain reaction; GDH = glutamate dehydrogenase; EIA = enzyme immunoassay.

and-toxin EIA, without resolution of discordant samples, had 57.6% sensitivity and 59.7% specificity (Table 1). After excluding samples from patients with a Bristol scale score of 4, specificity of PCR alone was 80.7%.

Algorithm 1, which reflexed discordant GDH+/toxin− samples ($n = 39$) to PCR, yielded 97.0% sensitivity and 79.0% specificity. Algorithm 2, which reflexed PCR+ samples to EIA toxin testing ($n = 45$), yielded 57.6% sensitivity and 100.0% specificity.

3.3. TcdA and TcdB concentration

A second goal of this study was to utilize the Clarity assay's ultrasensitivity to further understand the putative differences between samples reported as positive, negative, or undetermined by other tests and algorithms. When the samples were tested with algorithm 1, the median *C. difficile* toxin concentrations in GDH+/toxin+ ($n = 19$), GDH+/toxin−/PCR+ ($n = 26$), GDH+/toxin−/PCR− ($n = 13$), and GDH−/toxin− ($n = 37$) samples were 3589.5 (interquartile range [IQR] 795.5–19,326.0), 11.5 (IQR 1.74–79.6), 0.45 (IQR 0.15–3.0), and 0 (IQR 0–0.9) pg/mL ($P < 0.001$; Fig. 2a). Clarity results were positive in 19/19 GDH+/toxin+, 12/26 GDH+/ toxin−/PCR+, 1/13 GDH+/toxin−/PCR−, and 0/37 GDH−/toxin− samples. When the samples were tested with algorithm 2, the median *C. difficile* toxin concentrations in PCR+/toxin+ ($n = 19$), PCR+/toxin− ($n = 26$), and PCR− (n = 50) samples were 3589.5 (IQR 795.5–19,326.0), 11.5 (IQR 1.74–79.6), and 0.15 (0–1.0) pg/mL ($P < 0.0001$; Fig. 2b). Clarity results were positive in 19/19 PCR+/toxin+, 13/26 PCR+/toxin−, and 1/50 PCR− samples.

4. Discussion

Currently available tests for CDI diagnostics are limited by poor specificity or sensitivity, and/or long turnaround times. The Infectious Diseases Society of America (IDSA), the Society for Healthcare Epidemiology of America (SHEA), and the European Society of Clinical Microbiology and Infectious Diseases therefore recommend using multistep testing algorithms for laboratory confirmation of CDI diagnosis (Crobach et al., 2016; McDonald et al., 2018).

In this study, the laboratory performance of the Singulex Clarity C. diff toxins A/B assay, PCR, GDH-and-toxin EIA, and multistep algorithms compared to CCNA was determined. The Clarity assay had similar sensitivity but higher specificity than PCR, and higher sensitivity and specificity than a rapid-membrane GDH-and-toxin EIA. The Clarity assay also outperformed 2 multistep algorithms in both sensitivity and specificity, while generating results in under 35 min on the automated Clarity instrument. Testing with the GDH-and-toxin EIA (time to result: 30 min) resulted in discordance in 41.1% of the samples, and the specificity of PCR (time to result: 45 min) in the discordant sample set was only 48.0% compared to CCNA. This is in line with previous reports on the poor specificity of PCR (Burnham and Carroll, 2013; Crobach et al., 2016; Fang et al., 2017; McDonald et al., 2018; Planche et al., 2013; Polage et al., 2015). The discordant rate was higher than internal rates reported at TriCore Laboratories (approximately 15%; data not shown), which may partly be explained by the study design (not an all-comers study but a cohort with higher proportion of positive and discordant samples). Reflexing discordant EIA results to PCR yielded accuracy results similar to those observed when using PCR alone, and testing of PCR-positive samples with the toxin EIA had the same results as toxin EIA alone. Finally, testing with the PCR-plus-toxin algorithm reported 27.4% PCR+/toxin− samples, leaving clinicians with inconclusive results. This algorithm showed improved specificity over PCR alone but reduced the sensitivity to the same levels as toxin EIA (57.6%). IDSA and SHEA suggest that PCR can be used as a standalone test if clinicians and laboratorians agree not to submit stool specimens from patients receiving laxatives and to submit stool specimens only

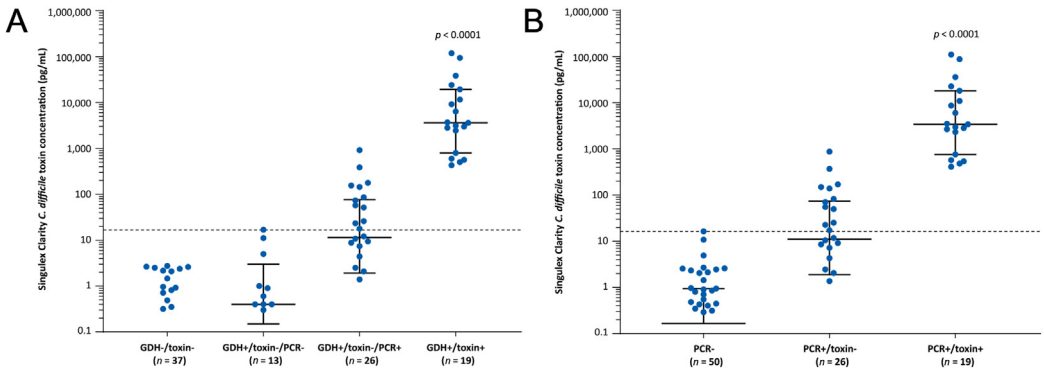

Fig. 2. Concentrations of TcdA and TcdB, as measured by the Singulex Clarity C. diff toxins A/B assay, in samples negative, discordant, and positive by (a) a 2-step algorithm utilizing GDH-and-toxin EIA (C. Diff Quik Chek Complete) arbitrated by PCR (BD MAX Cdiff Assay) and (b) a 2-step algorithm utilizing PCR testing with positive samples reflexed to toxin EIA. The dash line represents the preliminary cutoff for the Clarity assay. The central measurements represent median and whiskers quartile toxin concentrations. Abbreviations used: PCR = polymerase chain reaction; GDH = glutamate dehydrogenase.

from patients with unexplained and new onset on clinical diarrhea for testing for CDI (McDonald et al., 2018). However, when excluding samples from patients with Bristol scale 4, the specificity of PCR in this sample set did not improve significantly. In addition, 1 of the 50 PCR− samples was Clarity+/GDH+/CCNA+, indicating that a *tcdB* PCR might miss detection of a toxin-producing strain. In summary, these results highlight the complexity in interpreting multistep testing results for CDI diagnosis.

There is increasing evidence suggesting that the presence and detection of free toxins in stool better correlate with CDI severity and outcome than does the detection of toxigenic *C. difficile* (Bartlett and Gerding, 2008; Burnham and Carroll, 2013; Fang et al., 2017; McDonald et al., 2018;Planche et al., 2013; Polage et al., 2015). In this study, toxin concentrations in samples stratified by algorithm results were compared. Using the EIA-plus-PCR algorithm, there was a significant, gradual decrease in toxin concentration between GDH+/toxin+, GDH+/toxin−/PCR+, GDH+/toxin−/PCR−, and GDH−/toxin− samples. The same difference was observed in PCR+/toxin+, PCR+/toxin−, and PCR− samples. These findings indicate that measurements of TcdA and TcdB concentrations may have the potential to guide treatment by distinguishing between colonized individuals and CDI patients. In a recent study, it was reported that *C. difficile* toxin concentrations were significantly higher in CDI patients than in asymptomatic carriers of toxigenic *C. difficile*, but the difference was seen only when CDI was diagnosed by toxin detection and not when diagnosed by NAAT (Pollock et al., 2018). When testing CDI suspects and asymptomatic inpatients with PCR, the groups had a similar positivity rate (Truong et al., 2017), suggesting that some PCR+ results in symptomatic patients are possibly due to *C. difficile* colonization. Diagnosing CDI by detecting presence of toxigenic *C. difficile* alone is endorsed by guidelines when institutional criteria for stool submission are met (McDonald et al., 2018) but may not be the preferred testing method (Planche et al., 2013; Polage et al., 2015).

The Singulex Clarity system incorporates Single Molecule Counting, a technology that facilitates ultrasensitive counting, enabling the quantification of analytes down to picogram-per-milliliter levels (Adamson et al., 2018; Evans et al., 2018; Januzzi et al., 2018; McCarthy et al., 2018; Walter et al., 2017). Single Molecule Counting technology may allow for assessment of CDI severity, and possibly guide treatment, based on *C. difficile* toxin concentration. Individuals can carry toxigenic *C. difficile* without having CDI (Bartlett and Gerding, 2008; Burnham and Carroll, 2013; Kelly, 2012; Kelly and Kyne, 2011; Loo et al., 2011; McDonald et al., 2018), and the presence of TcdA and TcdB in low concentrations may correspond to colonization but not disease. Asymptomatic colonization has been reported to reinforce the adaptive immune response to *C. difficile*, which may protect against the bacteria, and higher levels of serum anti-TcdA and anti-TcdB antibodies may protect against CDI (Bauer et al., 2014; Furuya-Kanamori et al., 2015; Kelly and Kyne, 2011; Leav et al., 2010; Péchiné and Collignon, 2016). Both immunization and treatment with monoclonal antibodies against TcdA and TcdB have been shown to protect against CDI and recurrences (Henderson et al., 2017; Kelly and Kyne, 2011; Wilcox et al., 2017). Healthy adults commonly carry anti-TcdA and anti-TcdB antibodies, without being colonized by *C. difficile*, which is likely the result of colonization in infancy combined with subsequent exposures to the bacteria (Kelly and Kyne, 2011). Because the Clarity instrument and assay enable the quantitation across a large dynamic range, including toxins at very low concentrations, the assay may potentially allow for the distinction between nonpathogenic levels of toxins and levels associated with disease and might enhance our understanding of and protection against CDI.

5. Conclusions

The Singulex Clarity C. diff toxins A/B assay provided ultrasensitive detection of TcdA and TcdB, with high sensitivity and specificity compared to CCNA, and outperformed EIA, PCR, and multistep algorithms recommended by current guidelines. The Clarity assay may offer a standalone solution for CDI diagnostics, and measurements of TcdA and TcdB concentrations may have the potential to guide treatment and infection-control practices.

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