High Agreement Between an Ultrasensitive *Clostridioides difficile* Toxin Assay and a *C. difficile* Laboratory Algorithm Utilizing GDH-and-Toxin Enzyme Immunoassays and Cytotoxin Testing

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ABSTRACT The Singulex Clarity *C. diff* toxins A/B (Clarity) assay is an automated, ultrasensitive immunoassay for the detection of *Clostridioides difficile* toxins in stool. In this study, the performance of the Clarity assay was compared to that of a multistep algorithm using an enzyme immunoassay (EIA) for detection of glutamate dehydrogenase (GDH) and toxins A and B arbitrated by a semiquantitative cell cytotoxicity neutralization assay (CCNA). The performance of the assay was evaluated using 211 residual deidentified stool samples tested with a GDH-and-toxin EIA (*C. Diff* Quik Chek Complete; Techlab), with GDH-and-toxin discordant samples tested with CCNA. The stool samples were stored at -80°C before being tested with the Clarity assay. For samples discordant between Clarity and the standard-of-care algorithm, the samples were tested with PCR (Xpert *C. difficile*; Cepheid), and chart review was performed. The testing algorithm resulted in 34 GDH⁺/toxin⁺, 53 GDH⁻/toxin⁻, and 124 GDH⁺/toxin⁻ samples, of which 39 were CCNA⁺ and 85 were CCNA⁻. Clarity had 96.2% negative agreement with GDH⁻/toxin⁻ samples, 100% positive agreement with GDH⁺/toxin⁺ samples, and 95.3% agreement with GDH⁺/toxin⁻/CCNA⁻ samples. The Clarity result was invalid for one sample. Clarity agreed with 61.5% of GDH⁺/toxin⁻/CCNA⁺ samples, 90.0% of GDH⁺/toxin⁻/CCNA⁺ (high-positive) samples, and 31.6% of GDH⁺/toxin⁻/CCNA⁺ (low-positive) samples. The Singulex Clarity *C. diff* toxins A/B assay demonstrated high agreement with a testing algorithm utilizing a GDH-and-toxin EIA and CCNA. This novel automated assay may offer an accurate, stand-alone solution for *C. difficile* infection (CDI) diagnostics, and further prospective clinical studies are merited.

KEYWORDS *C. difficile*, *C. difficile* EIA, *C. difficile* PCR, CDI, single-molecule counting, cytotoxin, toxin, ultrasensitive

Clostridioides difficile (formerly *Clostridium difficile*) is an anaerobic, Gram-positive, spore-forming bacterium first identified in 1935 (1, 2). In the 1970s, the causative relationship between *C. difficile* infection (CDI) and antibiotic-associated pseudomembranous colitis was established, and CDI is now the most common cause of nosocomial diarrhea (3–6).

CDI is a clinical diagnosis, requiring the presence of symptoms, usually acute diarrhea, and identification of either *C. difficile* toxins A and/or B or toxigenic species in stool. *In vitro* diagnostic tests for CDI have either poor clinical specificity (nucleic acid amplification tests [NAATs]), poor sensitivity (toxin enzyme immunoassays [EIAs]), or

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long turnaround time (cell cytotoxicity neutralization assay [CCNA] and toxigenic culture [TC]) (7, 8). Consequently, current guidelines recommend multistep testing algorithms for CDI diagnosis (9, 10).

The Singulex Clarity *C. diff* toxins A/B assay (Clarity) is an automated and ultrasensitive assay, powered by single-molecule counting technology, for the detection of *C. difficile* toxins A and B in stool. In this study, the performance of the Clarity assay was compared to that of a standard-of-care algorithm using an EIA for detection of glutamate dehydrogenase (GDH) and toxins A and B arbitrated by CCNA.

MATERIALS AND METHODS

Singulex Clarity *C. diff* toxins A/B assay. The Clarity assay detects *C. difficile* toxins A and B in stool on the Singulex Clarity system, as has been described previously (11–14). Briefly, either 100 μ l of a liquid or semisolid or 0.1 g (not tested in this study) of a solid stool sample is mixed (1:20) with diluent buffer and centrifuged at $14,000 \times g$ for 10 min. Three hundred microliters of the resulting supernatant is loaded onto the Singulex Clarity system. The sample is mixed with paramagnetic microparticles pre-coated with antitoxin A and antitoxin B 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 toxin A-toxin B concentration. The limits of detection for toxins A and B are 0.8 and 0.3 pg/ml in buffer and 2.0 and 0.7 pg/ml in stool, respectively (11). The cutoff for the Clarity assay compared to CCNA, as stated in the manufacturer's instructions for use, is set at 12.0 pg/ml (13). Time to first result is 32 min.

Study design. Stool samples ($n = 211$) from patients with suspected CDI were tested and collected at Yale New Haven Hospital (YNHH) in New Haven, CT, from July to November 2018. One hundred sixteen (55.0%) of the patients were women, and 204 (96.7%) were age 18 years or older. In total, there were 194 unique patients included in the study; 13 patients had two samples and 2 patients had three samples entered. Samples were tested onsite within 2 to 12 h of collection with a rapid EIA for the detection of GDH and toxins A and B (*C. Diff* Quik Chek Complete [QCC], TechLab, Inc., Blacksburg, VA), and GDH-and-toxin discordant samples were tested with a semiquantitative CCNA using the *C. difficile* toxin/antitoxin kit reagents (catalog no. T5000; TechLab, Inc.). CCNA was performed by testing serial dilutions (1:10, 1:100, 1:1,000, and 1:10,000) of fecal filtrate of a 1:1 dilution of stool to phosphate-buffered saline (PBS) suspension, in 96-well plates of laboratory-prepared human foreskin fibroblast culture (Quidel, San Diego, CA).

Residual deidentified stool samples were stored at -80°C and shipped to Singulex (Alameda, CA) for testing with the Clarity assay. The performance of Clarity was compared to that of the standard-of-care algorithm. For samples discordant between Clarity and the standard-of-care algorithm, chart review was performed and the samples were tested with NAAT (Xpert *C. difficile*; Cepheid, Sunnyvale, CA) tested at YNHH, and with a second qualitative CCNA (*C. difficile* TOX-B test; catalog no. T5003, TechLab; tested at ARUP Laboratories, Salt Lake City, UT), which utilizes a 1:10-diluted fecal filtrate in PBS, tested at a single final dilution of 1:50.

RESULTS

Of 211 samples tested, the standard-of-care testing algorithm (YNHH) resulted in 34 QCC GDH⁺/toxin⁺ and 53 QCC GDH[−]/toxin[−] samples (Fig. 1). Among the 124 GDH⁺/toxin[−] samples that reflexed to CCNA testing, 39 were CCNA⁺ and 85 were CCNA[−], using the semiquantitative CCNA. Of the 73 total samples toxin⁺ by the standard-of-care algorithm, QCC detected 34 (46.6%), while Clarity detected all 34 QCC toxin⁺ and 24 of 39 CCNA⁺ samples, or a total of 58 of 73 toxin⁺ samples (79.5%). One GDH⁺/toxin[−]/CCNA[−] sample had invalid Clarity and NAAT results and was excluded from analysis. Thus, Clarity had 100% positive agreement with GDH⁺/toxin⁺ samples, 96.2% negative agreement with GDH[−]/toxin[−] samples, and 95.7% negative agreement with the standard-of-care algorithm. In addition, Clarity agreed with 95.3% of GDH⁺/toxin[−]/CCNA[−] samples.

Results were further analyzed by correlating Clarity values with semiquantitative CCNA results (Table 1 and Fig. 2). Clarity agreed with 18/20 (90.0%) of high-positive GDH⁺/toxin[−]/CCNA⁺ samples (dilution steps 1:1,000 to 1:10,000) and 6/19 (31.6%) of low-positive GDH⁺/toxin[−]/CCNA⁺ samples (dilution steps 1:10 to 1:100) samples. Clarity was also positive in three GDH⁺/toxin[−]/CCNA[−] samples and two GDH[−]/toxin[−] samples.

FIG 1 The standard-of-care testing algorithm (YNHH) and Clarity results. One GDH⁺/toxin⁻/CCNA⁻ sample had invalid Clarity and NAAT results and was excluded from analysis. Abbreviations: GDH, glutamate dehydrogenase; CCNA, cell cytotoxicity neutralization assay.

Analysis of discrepant samples. Twenty-one discrepant samples were tested by NAAT and by qualitative CCNA (Tables 2 and 3). All 15 GDH⁺/toxin⁻/CCNA⁺ samples that were negative by Clarity were positive by NAAT for the toxin B gene. However, only 11 of the 15 were positive for toxin by qualitative CCNA at ARUP. In particular, 3 of 7 CCNA⁺ at the 1:10 dilution at YNHH tested CCNA⁻ at a 1:50 final dilution at ARUP.

Chart reviews revealed the difficulty in making a clinical diagnosis of CDI as the cause of a patient's symptoms. Selected data from chart reviews are presented in Table 3. For 15 CCNA⁺/Clarity⁻ discrepant samples, 14 patients had prior hospital admissions and multiple comorbidities. Two patients with the highest CCNA titers, 1:1,000 and 1:10,000, appeared to have CDI and responded to therapy. Of note, their NAAT cycle threshold (C_T) values were higher than the cutoff of 27, which has been reported to correlate with the presence of free toxins (15–17). Of the other 12 patients with CCNA-positive results of 1:10 or 1:100, 3 had no diarrhea in the hospital. Indeed, two of these were constipated and had been given laxatives and one had a gastrointestinal (GI) bleed that could also account for loose stools. Two had chronic diarrhea, one was on tube feedings, and both had minimal or no response to treatment. Two were on laxatives, two were not treated, three died acutely of other causes, and two patients were transferred to hospice care.

Two GDH⁺/toxin⁻/CCNA⁻ samples positive by Clarity were NAAT⁺. One of these was also CCNA⁺ at ARUP, had a C_T of 22.6, and had a Clarity result of 35.2 pg/ml (cutoff, 12.0 pg/ml), yet the patient was not treated and recovered. The other patient with a NAAT⁺ (C_T 28.5) and Clarity⁺ (22.4) stool, but negative by CCNA at both YNHH and ARUP, had a GDH⁺/toxin⁺ sample 8 days later and was then treated for CDI. In addition, three Clarity⁺ samples were negative by both NAAT and qualitative CCNA.

DISCUSSION

Making a timely and accurate laboratory diagnosis of CDI remains a challenge. Toxigenic culture and NAATs target the *C. difficile* toxin gene and detect toxigenic

TABLE 1 Correlation of semiquantitative CCNA (YNHH) titers and Singulex Clarity results^a

CCNA dilution factor	Singulex Clarity <i>C. diff</i> toxins A/B assay		
	Positive	Negative	Total
10	0	7	7 ^b
100	6	6	12
1,000	8	1	9
10,000	10	1	11
Total	24	15	39

^aCell cytotoxicity neutralization assay (CCNA) at YNHH was performed by testing serial dilutions (1:10, 1:100, 1:1,000, and 1:10,000) of a 50% stool–PBS filtrate.

^bOnly 3 of 7 were positive by qualitative CCNA at ARUP.

FIG 2 *C. difficile* toxin concentration in samples in various result categories. Combined toxin A and B concentrations are shown for stool samples with different results by GDH-and-toxin EIA and a semiquantitative CCNA (dilution steps, 1:10, 1:100, 1:1,000, and 1:10,000). The dotted line represents the cutoff for the Clarity assay (12.0 pg/ml).

bacteria and cannot differentiate a carrier state from active disease (18–20). CCNA, a gold standard method since its introduction in 1978 (3), has been shown in recent reports to correlate with mortality, in contrast to NAAT (18), but requires cell culture expertise, use of sensitive cell monolayers, and 24 to 48 h of incubation; thus, it is rarely used for routine diagnosis. The most common method to detect free toxin in stool, toxin EIA, has suboptimal sensitivity (7). Thus, more sensitive, rapid, and user-friendly toxin assays are urgently needed.

In this study, the Singulex Clarity *C. diff* toxins A/B assay was found to be significantly more sensitive than the QCC toxin EIA, detecting all samples ($n = 34$) positive by QCC and 61.5% (24 of 39) of the samples positive only by the semiquantitative CCNA. In addition, Clarity detected two additional likely true positives missed by CCNA at

TABLE 2 Comparison between the Clarity toxin assay and the standard-of-care algorithm utilizing a GDH-and-toxin EIA and a semiquantitative CCNA^a

	Standard-of-care algorithm (YNHH)				Arbitration of discrepant results	
No. with indicated result	QCC GDH	QCC toxin	Reflex to CCNA	Singulex Clarity	Toxin B gene NAAT	CCNA ARUP
Toxin positive by algorithm (n = 73)						
34	Pos	Pos	ND	Pos	ND	ND
24	Pos	Neg	Pos	Pos	ND	ND
15 ^b	Pos	Neg	Pos	Neg	Pos	11/15 pos
Toxin negative by algorithm (n = 137)						
81 ^c	Pos	Neg	Neg	Neg	ND	ND
2 ^d	Pos	Neg	Neg	Pos	Pos	1/ 2 Pos
1	Pos	Neg	Neg	Pos	Neg	Neg
51	Neg	Neg	ND	Neg	ND	ND
2	Neg	Neg	ND	Pos	Neg	Neg

^aQCC, *C. Diff* Quick Chek Complete; GDH, glutamate dehydrogenase; Pos, positive; Neg, negative; ND, not done.

^bOnly 11 of 15 were positive by qualitative CCNA at ARUP.

^cOne sample invalid by both Clarity and NAAT was excluded.

^dBoth were NAAT positive; one of two was positive by CCNA at ARUP. The other patient became QCC toxin positive 8 days later.

TABLE 3 *C. difficile* discrepant test result chart review^a

Result and study ID ^b CCNA ⁺ /PCR ⁺ /Clarity ⁻	Age	Host	Significant diarrhea	History of CDI	Laxative	CDI Rx	Response to Rx	Follow-up	CCNA YNHH	PCR C _T value	CCNA ARUP	Clarity
CD2-189	75	ESRD, abdominal pain, diarrhea	Large stool in rectum; given mineral oil to pass stool	Y	Y	Y	No further diarrhea	Negative tests	10	29.6	Neg	10.9
CD2-104	88	Dementia, stroke, PNA	Liquid stool	N	N	Y	No response	Transfer to comfort care; deceased	10	30.8	Neg	3.9
CD-44	87	Lives in ECF. Melena, reported diarrhea, given loperamide (Imodium)	No diarrhea in hospital; formed stool tested	Y	N	Y	No diarrhea in hospital	Doing well	10	33	Neg	7.1
CD2-209	74	Fell at home, COPD, CKD, GI bleed, history of CDI	GI bleed	Y	N	Y	GI bleed resolved	WBC remained elevated for wks	10	34.7	Neg	4
CD2-190	81	Stroke, PNA	Liquid stool	N	N	Y	Died acutely	NA	10	34.6	Pos	5.9
CD2-48	87	Metastatic CA	None. Given laxative for constipation.	N	Y	N	Not treated	Transfer to Hospice; deceased	10	35.1	Pos	2.9
CD2-132	58	Metastatic prostate CA, diverticulitis, ETOH withdrawal, suicide attempt	Intermittent loose stools. Prior result [CD2-09] noted and treated	N	N	Y	Not stated	No further tests	10	28.5	Pos	4.4
CD2-09	58	Metastatic prostate CA, diverticulitis, ETOH withdrawal	Intermittent. Asymptomatic at discharge. Cytotoxin result not seen.	N	N	N	Not treated	Readmitted. See CD2-132.	100	30	Pos	6.3
CD2-07	64	Fall, aspiration PNA	Liquid stool	N	N	Y	Died acutely	Deceased	100	21.2	Pos	8.3
CD2-211	44	ESRD, BKA stump infection	Chronic diarrhea, no clear etiology despite prior colonoscopy. Discharged before finishing treatment.	Y	N	Y	Readmitted 2 mo later, again reported chronic diarrhea, <i>C. difficile</i> tests negative	Subsequent <i>C. difficile</i> tests negative	100	25.3	Pos	8.6
CD2-165	78	Surgery, pituitary adenoma, intracranial bleed, PNA	Loose stools, incontinent. On tube feeds.	N	Y	Y	Some diarrhea continued, but <i>C. difficile</i> test negative	Subsequent <i>C. difficile</i> tests negative	100	25.7	Pos	4.1
CD2-78	80	Metastatic prostate CA, ESRD, dialysis, UTI, WBC increased to 37,000 on antibiotics	Details not recorded	Y	Y	Y	Notes state diarrhea improved, WBC fell	Repeat <i>C. difficile</i> test while on therapy positive	100	29.5	Pos	4.4
CD2-22	71	Respiratory failure	Watery stools x 2 d	N	N	Y	Died acutely from respiratory failure	Deceased	100	32.9	Pos	3.8
CD2-108	89	Admission diagnosis: sepsis due to CDI	Liquid stools	N	N	Y	Resolved	None	1,000	31.6	Pos	9.8
CD2-80	88	Lives in ECF. ESRD, PNA, diarrhea	Diarrhea, abdominal pain	Y	N	Y	Resolved	None	10,000	27.8	Pos	6

(Continued on next page)

TABLE 3 (Continued)

Result and study ID ^a CCNA-/PCR+/Clarity ⁺	Age	Host	Significant diarrhea	History of CDI	Laxative	CDI Rx	Response to Rx	Follow-up	CCNA YNH	PCR C _r value	CCNA ARUP	Clarity
CD2-171	77	Cirrhosis. On antibiotics for PNA.	Slightly worse than baseline	Y	N	N	Not treated	Discharged without therapy. No further <i>C. difficile</i> tests.	Neg	22.6	Pos	35.2
CD2-179	74	Cervical CA, BO, ileostomy	Ostomy output increased, slight blood	Y	Y	N	Not treated	Rapid toxin positive 8 days later, treated	Neg	28.5	Neg	22.4
CCNA-/PCR-/Clarity ⁺ CD2-30	55	MI, MVR, VAP	Not stated	N	Y	N	Not treated	No diarrhea or further testing	ND (GDH neg)	Neg	Neg	14.8
CD2-81	51	Lymphoma, transplant	No. Copious formed stool in bowel.	N	N	N	Not treated	<i>C. difficile</i> tests negative	ND (GDH Neg)	Neg	Neg	14.9
CD2-17	64	Outpatient. Esophageal reflux, Barrett's esophagus, IBS	Reported diarrhea for 2 wks	N	N	Y	Treated inappropriately based on GDH ⁺ only	No other diarrhea or <i>C. difficile</i> testing	Neg	Neg	Neg	16.6

^aAbbreviations: ID, identifier; CDI, *C. difficile* infection; Rx, treatment; YNH, Yale New Haven Hospital; C_r, cycle threshold; ARUP, ARUP reference laboratory; Y, yes; N, no; NA, not applicable; ESRD, end-stage renal disease; CA, cancer; PNA, pneumonia; ECF, extended-care facility; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; ETOH, ethanol; BKA, below-knee amputation; UTI, urinary tract infection; BO, bowel obstruction; MI, myocardial infarction; MVR, mitral valve replacement; VAP, ventilator-associated pneumonia; IBS, irritable bowel syndrome; WBC, white blood cell.

^bCCNA performed at YNH.

YNHH. Clarity toxin concentration had a strong linear correlation with semiquantitative CCNA titers.

The semiquantitative CCNA used in this study was shown to be more sensitive than the qualitative CCNA available at commercial reference laboratories and used in a previous study (11). Of the 15 semiquantitative CCNA⁺/Clarity[−] samples, 13 were low positives and for many patients, the diagnosis of CDI could be questioned due to presence of other possible causes of diarrhea, absence of diarrhea, failure to respond to treatment, and/or chronic diarrhea with subsequent negative CDI test results. Although all CCNA⁺ samples were also NAAT⁺, and thus toxigenic bacteria were present, it has been shown that free toxin can also be found in patients without CDI (21), and a low-positive CCNA might therefore not indicate that *C. difficile* is the cause of the patient's symptoms. While it is possible that the Clarity result may actually correlate better with CDI, further prospective clinical studies are needed to answer this question.

In this study, there were also 3 samples positive by Clarity and negative by both NAAT and qualitative CCNA. However, the NAAT assay detects the presence of the toxin B gene and CCNA primarily detects the presence and activity of toxin B (7), while the Clarity assay detects both toxins A and B. However, these Clarity⁺ samples were not tested for toxin A, and two were GDH negative and thus negative for *C. difficile* bacteria. Thus, although not applicable in these cases, it should be noted that some Clarity-positive samples could potentially be explained by detection of toxin A. The role of the different toxins in CDI has been debated (22), and recent studies have shown that strains predominantly producing toxin A are relevant in CDI and can cause disease (23–25). Unfortunately, determination of the prevalence of toxin A, toxin B, and toxins A and B among the samples tested was beyond the scope of the study.

This study highlights the challenges in accurately diagnosing CDI in symptomatic patients. CDI is a toxin-mediated disease; due to the high incidence of both diarrhea and colonization in tested populations, the detection of toxigenic organisms leads to overdiagnosis and overtreatment (7, 18, 19). In a recent study, it was shown that presence of toxins, as measured with the Clarity assay, correlated with CDI relapse, death, and severity of disease and that the proportion of CDI overdiagnosis was more than three times higher in NAAT⁺/toxin[−] than in NAAT⁺/toxin⁺ patients (14). Use of the Clarity ultrasensitive toxin assay improved clinical specificity compared with that of NAAT, without the poor sensitivity shown by contemporary toxin EIAs (14).

In summary, the Singulex Clarity *C. diff* toxins A/B assay demonstrated high agreement with a testing algorithm utilizing a GDH-and-toxin EIA and a semiquantitative CCNA, and there was a strong linear correlation between Clarity toxin concentration and semiquantitative CCNA results.

While the novel, automated Clarity toxin assay may offer an accurate, stand-alone solution for CDI diagnostics, at the time of publication of this article, Singulex has chosen not to pursue commercial application of the Clarity assay. Nevertheless, our results shed further light onto the potential of single-molecule counting technology for free-toxin detection, which should continue to inform *C. difficile* diagnostics.

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