

Comparison of *C. difficile* stool toxin concentrations in adults with symptomatic infection and asymptomatic carriage using an ultrasensitive quantitative immunoassay

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Running title: Toxin concentrations in CDI vs carriage

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Summary: We compared stool toxin concentrations in adult inpatients with symptomatic CDI versus asymptomatic carriage. Median concentrations differed only when CDI was defined by detectable stool toxin (versus positive NAAT); concentration did not differentiate an individual with CDI from a carrier.

**Abstract:**

Background: We used an ultrasensitive, quantitative Single Molecule Array (Simoa) immunoassay to test whether concentrations of *C. difficile* toxins A and/or B in the stool of adult inpatients with CDI were higher than in asymptomatic carriers of toxinogenic *C. difficile*.

Methods: Patients enrolled as CDI-NAAT had clinically significant diarrhea and positive nucleic acid amplification testing (NAAT), per US guidelines, and received CDI treatment. Potential carriers had recently received antibiotics and did not have diarrhea; positive NAAT confirmed carriage. Baseline stool samples were tested by Simoa for toxin A and B.

Results: Stool toxin concentrations in both CDI-NAAT (n = 122) and Carrier-NAAT (n =44) cohorts spanned five logs (0 pg/mL to >100,000 pg/mL). 79/122 (65%) CDI-NAAT and 34/44 (77%) Carrier-NAAT had toxin A+B concentration  $\geq 20$  pg/mL (clinical cutoff). Median toxin A, toxin B, toxin A+B and NAAT Ct values in CDI-NAAT and Carrier-NAAT cohorts were similar (toxin A, 50.6 vs 60.0 pg/mL, p=0.959; toxin B, 89.5 vs 42.3 pg/mL, p=0.788; toxin A+B, 197.2 vs 137.3 pg/mL, p=0.766; Ct, 28.1 vs 28.6, p=0.354). However, when CDI/Carrier cohorts were limited to those with detectable toxin, respective medians were significantly different (A, 874.0 vs 129.7, p=0.021; B, 1317.0 vs 81.7, p=0.003, A+B, 4180.7 vs 349.6, p=0.004; Ct, 25.8 vs 27.7, p=0.015).

Conclusions: Toxin concentration did not differentiate an individual with CDI from one with asymptomatic carriage. Median stool toxin concentrations in groups with CDI versus carriage differed, but only when groups were defined by detectable stool toxin (versus positive NAAT).

Keywords: *C. difficile*, toxin, Simoa, carriage, diarrhea

## Introduction

The international medical community has not reached consensus regarding optimal strategies for diagnosis of *Clostridioides* (formerly *Clostridium*) *difficile* infection (CDI) [1]. The wide variability in clinical presentation and outcomes of CDI (ranging from mild diarrhea to severe colitis and death) [2], combined with the potential for asymptomatic carriage (presence of toxinogenic *C. difficile* in the colon but no symptoms of CDI), have generated considerable diagnostic confusion. Despite decades of investigation and debate regarding the relative merits of tests capable of detecting either toxinogenic *C. difficile* bacteria [e.g. culture or nucleic acid amplification testing (NAAT)] or the toxins it can produce [cytotoxicity assay (CTA) or enzyme immunoassay (EIA)] [2], it remains unclear which tests or algorithmic combinations of tests provide highest diagnostic utility for CDI [1, 3]. Meanwhile, CDI marches forward as a top etiology of nosocomial bacterial infection in the United States, with high cumulative morbidity, mortality, and cost [3, 4].

Some experts recommend prioritizing toxin detection to guide treatment decisions, based on data suggesting that patients with detectable toxin in stool (compared to patients whose stool is NAAT-positive for a toxin gene, but is toxin-negative) are at highest risk for poor outcomes [5, 6]. However, current toxin detection tests are considered either too analytically insensitive (EIA) or too cumbersome and slow (CTA) to provide optimal utility. Importantly, the arguments in support of the prognostic value of toxin detection are confounded by studies that show that toxin can sometimes be detected in the stool of asymptomatic individuals [7, 8]. However, no study thus far has, to our knowledge, quantified and directly compared stool toxin concentrations in parallel cohorts of untreated patients who are

carriers of (asymptomatic) versus infected with (symptomatic) toxinogenic *C. difficile*. In part, this knowledge gap remains because there previously was no assay capable of highly sensitive, quantitative and separate measurements of *C. difficile* toxins A and B over the necessary concentration ranges.

We have recently developed an ultrasensitive assay for detection and quantification of *C. difficile* toxins utilizing Single Molecule Array (Simoa) technology [9, 10]. This assay is capable of separately detecting and quantifying *C. difficile* toxins A and B over a five-log range of concentrations, starting from an analytical cutoff of approximately 1 pg/mL and clinical cutoff of approximately 20 pg/mL in diluted stool samples. Given the current impasse in the field regarding detection of toxin versus organism, we set out to use a next-generation Simoa assay to directly compare concentrations of toxins A and B in the stool of NAAT+ adult inpatients with CDI (with or without the presence of detectable toxin) versus those with asymptomatic *C. difficile* carriage. Our a priori primary hypothesis was that concentrations of *C. difficile* toxin A and/or B would be higher in the stool of individuals with CDI as compared to symptomless carriers of toxinogenic *C. difficile*.

## Methods

CDI-NAAT cohort: Eligible patients were inpatients  $\geq 18$  years old with positive clinical stool NAAT result (below), were initiating CDI therapy, and had diarrhea, defined as follows: a) documentation of three or more unformed bowel movements during any 24 hours in the 48 hours before stool collection, OR b) persistent diarrhea in the 48 hours before stool collection per medical notes. Whenever possible, definition “a” was applied. Assessment for the presence of diarrhea included review of nursing input/output logs for number and consistency of stools, consultation with treating clinicians, and

detailed chart review (requiring mention of “diarrhea,” “loose stools,” and/or increased frequency, in notes written by multiple providers). Patients for whom there was any doubt about the presence of diarrhea, or who had chronic diarrhea, were excluded. The diagnostic clinical stool sample (submitted for routine *C. difficile* testing) was captured as a discarded sample. Patients were excluded if the diagnostic specimen was of insufficient volume or >72 hours old, if they had received CDI treatment for >24 hours prior to stool collection, or if they had a colostomy. Administration of any laxative in the 72 hours prior to sample collection was recorded. Peak WBC count/creatinine and nadir albumin values within five days before to two days after stool collection were recorded. ICU admission, colectomy, and death rates were assessed during the 40 days after enrollment.

Asymptomatic Carrier-NAAT cohort: Eligible patients were inpatients  $\geq 18$  years old who were admitted for at least 72 hours, had received at least one dose of an antibiotic within the past seven days, and had absence of diarrhea, defined as follows: no report of diarrhea by patient or nurse in the 48 hours prior to enrollment (absence of diarrhea in the preceding 48 hours was re-confirmed at time of stool specimen receipt, if delayed relative to enrollment.). Assessment for the absence of diarrhea included detailed chart review, review of nursing input/output logs, and conversations with nurses and patients. Patients with 2 or more loose stools within a 24-hour period were excluded; patients with 1 loose stool were included only if providers had recently administered a laxative. Patients were excluded if they were unable to provide a stool sample, had a colostomy, received metronidazole PO/IV, vancomycin PO, rifaximin PO, and/or fidaxomicin PO for >24h within the past seven days, had been diagnosed with CDI in the past six months, or had tested negative for *C. difficile* within the past seven days. Recording of laxative receipt, laboratory measurements, and outcomes mirrored those for the CDI-NAAT cohort.

Subjects were followed for 40 days after stool collection for possible CDI occurrence. Stool samples were collected prospectively under verbal informed consent. NAAT (Xpert C. difficile/Epi) was performed on all samples, and positive samples retained as the Carrier-NAAT cohort.

*Details of sample handling, Simoa assay, NAAT, cytotoxicity assay, and statistical analysis are in Supplementary Materials.*

## **Results**

For the CDI-NAAT cohort, 122 samples met all inclusion/exclusion criteria. 44 of 382 (11.5%) stool samples from asymptomatic subjects were NAAT-positive (Carrier-NAAT cohort). The CDI-NAAT and Carrier-NAAT cohorts were demographically similar (Table 1), with no significant differences in baseline WBC, creatinine, or albumin values, nor rates of ICU admission, all-cause mortality, or colectomy within 40 days of enrollment. CDI-NAAT patients were more likely than Carrier-NAAT patients to test positive for a NAP-1 strain (12.3% versus 4.6%,  $p = 0.24$ ) (Table 1). 79/122 (65%) CDI-NAAT and 34/44 (77%) Carrier-NAAT subjects had total toxin (toxin A + toxin B) concentrations above a clinical cutoff of 20 pg/mL (Methods); these subgroups were designated as “CDI-Tox20” and “Carrier-Tox20” cohorts, respectively. CDI-Tox20 and Carrier-Tox20 cohorts were also similar to each other across all parameters (Table 1) with two exceptions: albumin levels were significantly lower in the CDI-Tox20 group (2.9 versus 3.4 mg/dL,  $p = 0.017$ ), and more CDI-Tox20 subjects tested positive for NAP-1 (19.0% versus 5.8%,  $p = 0.09$ ).

Toxin A and B concentrations as measured by Simoa in diluted stool (21-fold dilution factor) were distributed over a five-log range (0 to >100,000 pg/mL). Stool toxin concentrations (as measured by Simoa) and Ct values (as measured by Xpert NAAT) in CDI-NAAT vs Carrier-NAAT cohorts are shown in Figure 1. Median values for toxin A concentration, toxin B concentration, and toxin A+B concentration were not significantly different in the CDI-NAAT and Carrier-NAAT cohorts (Table 2). In contrast, when the CDI-Tox20 and Carrier-Tox20 cohorts were compared, all median toxin concentration values (A, B, and A+B) were significantly higher in the CDI-Tox20 cohort (Figure 2, Table 2). Cytotoxicity assay (CTA) was performed on all samples; 60/122 (49%) CDI-NAAT and 17/44 (39%) Carrier-NAAT samples tested positive (designated CDI-CTA and Carrier-CTA cohorts). 55/59 (93%) CDI-NAAT and 13/14 (93%) Carrier-NAAT samples with Simoa toxin B concentrations  $\geq 100$  pg/mL had positive CTA results, whereas the majority of samples with toxin B concentrations <100 pg/mL tested negative by CTA (90% overall). 50/122 (41%) CDI-NAAT and 11/44 (25%) Carrier-NAAT patients had total toxin concentrations above 1000 pg/mL (as a proxy for EIA LOD; designated CDI-Tox1000 and Carrier-Tox1000 cohorts). CDI-CTA/Carrier-CTA and CDI-Tox1000/Carrier-Tox1000 groups were similar to each other across all parameters (Supplementary Table 1), with the exception that the % of CDI-CTA patients with WBC  $\geq 15$  was significantly higher than in the Carrier-CTA group (45.0% vs 17.7%,  $p = 0.051$ ). Median toxin concentrations were higher in CDI-CTA (versus Carrier-CTA) and CDI-Tox1000 (versus Carrier-Tox1000) cohorts (Supplementary Table 2), though differences were not statistically significant.

There was no toxin A+B concentration cutoff value that yielded useful sensitivity and specificity for distinction of CDI-NAAT versus Carrier-NAAT; the area under the receiver operator curve (AUC) was

0.515. Similarly, no cutoff adequately distinguished between CDI-Tox20 and Carrier-Tox20 (AUC 0.677), CDI-CTA and Carrier-CTA (AUC 0.651), or CDI-Tox1000 and Carrier-Tox1000 (AUC 0.631) groups.

Within 40 days of stool collection 3/44 (6.8%) of the Carrier-NAAT patients were suspected clinically to have CDI. However, only 1 fulfilled our study diagnostic criteria for CDI (see supplementary results for details). None of the remaining Carrier-NAAT patients were suspected to have CDI during the followup period, including 10 patients with toxin A+B concentrations >1000 pg/mL (four of whom had concentrations >10,000 pg/mL).

Median toxin A, toxin B, and toxin A+B values were compared for groups of stool samples of different consistencies (formed, semi-formed, liquid) in the CDI-NAAT and carrier-NAAT cohorts (Table 3). All median toxin concentrations trended up as stool consistency varied from formed to semi-formed to liquid, and Ct values trended down (Table 3). Proportions of each subgroup who had received any laxative within the 72 hours prior to stool collection are also noted in Table 3.

Median NAAT Ct values were not significantly different in the CDI-NAAT versus Carrier-NAAT cohorts, but significantly lower in the CDI-Tox20 versus the Carrier-Tox20 cohort (Fig 1D, Fig 2D, Table 2). Differences between NAAT Ct values in CDI-CTA/Carrier-CTA and CDI-Tox1000/Carrier-Tox1000 cohorts did not reach statistical significance (Supplementary Table 2). Xpert Ct values correlated moderately with toxin A, toxin B, and toxin A+B concentrations, and toxin A and toxin B concentrations correlated moderately with each other, in CDI-NAAT, Carrier-NAAT, CDI-Tox20, and Carrier-Tox20 cohorts

(Supplementary Table 3). Plots of these correlations for the CDI-NAAT and CDI-Tox-20 cohorts are shown in Figures 3 and 4, respectively.

## Discussion

Symptoms and signs consistent with CDI are highly variable, ranging from mild diarrhea to severe colitis, colectomy or death [2]. Provider concern about severe CDI-related outcomes is high enough that even patients with minimal or self-resolving diarrhea are sometimes tested and treated for CDI (e.g. [11]). Confusing the matter further, patients with no diarrhea whatsoever can still be colonized with toxinogenic *C. difficile*, and can transmit that organism to others who might be more susceptible to disease [12, 13]. It is no surprise that the medical community is struggling to decide between diagnostic algorithms focused on the most sensitive detection of toxinogenic organism in stool and algorithms focused on detection of actual *C. difficile* toxins in stool [1].

While the medical community has a shared goal—to detect patients who should be treated for CDI to prevent severe outcomes—there is disagreement about which patients who test positive for toxinogenic *C. difficile* should be treated. US guidelines [3, 14] focus on sensitive detection of toxinogenic organism (NAAT or GDH/toxin EIAs). For GDH+/toxin- EIA results, follow-up NAAT has been recommended [3, 15], suggesting that NAAT-positive results in symptomatic patients are to be considered diagnostic of CDI, even if results of currently available toxin tests are negative. In contrast, guidelines from Europe [16] prioritize detection of toxin, based on data suggesting that toxin-positive patients are at higher risk for bad outcomes [5, 6].

To date, the fact that current commercially available toxin immunoassays have limited analytical sensitivity (typical LOD ~1 ng/mL; [17, 18]) has significantly confounded diagnostic algorithms for CDI; importantly, it is clear that detection of toxin by EIA does not capture all patients at risk for severe CDI and its complications [19, 20]. This diagnostic gap led our group to develop ultrasensitive rapid toxin detection assays based on Single Molecule Array (Simoa) technology. With this technology, we were previously able to separately detect and quantify toxins A and B in stool with analytical LODs of < 1 pg/mL and clinical cutoffs of approximately 20 pg/mL in stool [9, 10]. In those studies, ~25% of NAAT-positive clinical samples had toxin B concentration <20 pg/mL by Simoa, but subjects were not rigorously screened to confirm that they had diarrhea. Here, we have demonstrated the application of Simoa assays to compare toxin concentrations in two rigorously defined NAAT-positive cohorts of adult inpatients within one hospital: patients with diarrhea consistent with CDI versus those who have no diarrhea or other clinical evidence of colitis. The hypothesis underlying this comparison was that patients with CDI (as diagnosed by NAAT, in combination with diarrhea) would have significantly higher stool toxin levels than asymptomatic carriers, and further, that the majority of asymptomatic carriers would have minimal or undetectable stool toxin. Secondly, we compared toxin concentrations in the subsets of these cohorts who had detectable stool toxin ( $A+B \geq 20$  pg/mL by Simoa). Our results have surprisingly but clearly demonstrated that stool toxin A and B concentration alone cannot distinguish a patient with CDI (diagnosed by either NAAT or toxin detection) from an asymptomatic carrier, because concentration distributions in both types of patients overlap substantially. However, our results also demonstrate that when considered as a group, toxin concentrations are significantly higher (and Ct values significantly lower) in CDI patients than in carriers—but this difference is seen only when CDI is diagnosed by toxin detection, and not when CDI is diagnosed by NAAT. Similarly, comparison of CDI and carrier cohorts defined by positive CTA, or by toxin  $A+B \geq 1000$  pg/mL by Simoa (as a proxy for the LOD of EIA), showed that toxin concentrations were higher (and Ct values lower) in CDI cohorts, though

reduced sample sizes likely affected the statistical significance. Our findings add strength to the argument that detection of toxin is more clinically relevant than detection of the toxin gene. While a prior study [21] did compare mean Ct values in symptomatic versus asymptomatic adult inpatients and find that they were similar, the symptomatic patients in that study were not evaluated to confirm that they actually had diarrhea consistent with CDI and, importantly, no toxin tests were performed.

There is recent interest in whether NAAT Ct values correlate with the presence or absence of detectable toxin (by EIA or CTA) in stool from symptomatic patients [22-24], potentially allowing use of NAAT results to predict which patients have elevated stool toxin levels and should thus be prioritized for treatment; other studies have investigated whether Ct value can be used to predict clinical outcome [25]. Senchyna et al [22] estimated that an Xpert Ct cutoff of 26.4 had a negative predictive value of 97.1% for excluding the presence of toxin in stool; however, as shown in Figures 3 and 4, multiple patients in our CDI-NAAT and CDI-Tox20 cohorts with Xpert Ct values >26.4 had detectable stool toxin by Simoa, including above analytical thresholds for EIA (~1000 pg/mL) and CTA (toxin B of ~100 pg/mL).

Given these findings, we may assume that neither stool toxin concentration nor Ct value can reliably distinguish a symptomatic patient with CDI from a symptomatic patient who is colonized and whose diarrhea is actually due to another cause.

Our findings beg the critical question of why some patients with high concentrations of *C. difficile* toxins in their stool are symptomatic and others are not. The demographics of our symptomatic and

asymptomatic patients were not significantly different. In terms of bacterial virulence, a higher percentage of the symptomatic patients with CDI had NAP-1 strains in stool, but at most, NAP-1 strains accounted for 19% of the strains in the CDI-Tox20 cohort. Given the likelihood that multiple factors in addition to toxin influence symptomatic expression of CDI, a full investigation of possible explanations will need to include analysis of host blood and stool inflammatory markers, blood and stool anti-toxin immunoglobulin levels, stool metabolites, multiplexed testing for other stool pathogens, and/or microbiome analyses to attempt to define key determinants of CDI versus the carrier state.

One potential limitation of our study was our requirement that asymptomatic patients have received at least one dose of antibiotic prior to enrollment, which was intended to increase the proportion of *C. difficile* carriers (given that antibiotic use is a risk factor for *C. difficile* colonization [26]). However, it is possible that this requirement selected for a population that was closer on the spectrum to the disease state (CDI) than a completely randomly-selected group of inpatients might have been. While we are certain that subjects enrolled as potential carriers did not have clinical diarrhea (Methods), we did observe that 28/44 (64%) of the carrier study stools were semi-formed or liquid (though we also note that the majority of the cohort had received laxatives; Table 3). Interestingly, stool consistency appeared to correlate with toxin concentration, with higher median toxin concentrations (and, to a lesser extent, lower Ct values) trending with less-formed stool. It is thus possible that some of the carrier patients had subclinical toxin-associated colitis, leading to higher water content in stool. Notably, however, only 1/44 carrier subjects progressed to probable CDI within 40 days of follow up. Thus, our findings suggest yet another, previously unrecognized, category in the clinical spectrum of CDI, i.e. subclinical colitis. Another limitation was that we were not able to fully control sample temperature between provision of a sample by the patient and the sample's arrival in the microbiology lab (at which

point it was refrigerated), which may have impacted toxin concentration. However, sample handling was the same for both CDI and carrier cohorts, and mirrored institutional sample handling for clinical *C. difficile* testing. Finally, because it was not possible to perform all tests in real time, we performed all testing (other than Xpert NAAT performed for initial clinical diagnosis) on frozen sample aliquots. We were fastidious in our refrigeration, mixing of samples prior to aliquoting, and weighing stool to ensure precise Simoa measurements despite variation in stool consistency [21], and all samples from both cohorts were processed in parallel using identical protocols and procedures. Finally, we recognize that results from a single center may not be generalizable.

Given that the presence of toxin is necessary, though not sufficient, for CDI, ultrasensitive measurement of stool toxin (rather than toxinogenic organism, by NAAT) may provide benefit by excluding CDI in symptomatic patients who are NAAT-positive but do not have detectable toxin, and potentially by supporting diagnosis and outcome prediction (currently under study) by providing an actual toxin concentration. However, if an additional institutional goal is to detect all patients who have *C. difficile* infection OR carriage to optimize infection control measures (since colonized patients can still transmit the organism to others [12, 13]), NAAT would likely be required, as there are NAAT-positive patients who do not have detectable stool toxin by Simoa. Ultimately, it may be that an accurate diagnosis of CDI will require both toxin measurement and additional testing for host biomarkers that can be shown to be specific to CDI; it is clear that clinically symptomatic CDI is the result of an interplay of multiple factors including host immune responses. Regardless, our data strongly reinforce the conclusion that accurate diagnosis of CDI cannot be done without rigorous confirmation of CDI symptoms—and consideration of all alternative explanations for those symptoms—prior to testing and treatment.

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## Conflict of Interest Declaration

A.B., B.R., A.L., and M.A.M. are employees of bioMerieux.

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All other authors have no conflicts of interest to declare.

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**Table 1****Comparison of demographics, baseline lab values, and clinical outcomes for CDI and carrier cohorts**

<b>Variable</b>	<b>CDI-NAAT (n=122)</b>	<b>Carrier-NAAT (n=44)</b>	<b>p- value</b>	<b>CDI-Tox20 (n = 79)</b>	<b>Carrier-Tox20 (n = 34)</b>	<b>p- value</b>
Age, median (IQR)	67.0 (55.0, 76.0)	64.5 (49.0, 73.5)	0.365	68.0 (57.0, 77.0)	65.5 (52.0, 77.0)	0.353
<b>Sex</b>			0.860			0.682
Female, N (%)	67/122 (54.9)	23/44 (52.3)		44/79 (55.7)	17/34 (50.0)	
Male, N (%)	55/122 (45.1)	21/44 (47.7)		35/79 (44.3)	17/34 (50.0)	
<b>Ethnicity</b>			0.454			0.435
Hispanic, N (%)	8/111 (7.2)	1/36 (2.8)		7/71 (9.9)	1/28 (3.6)	
Not Hispanic, N (%)	103/111 (92.8)	35/36 (97.2)		64/71 (90.1)	27/28 (96.4)	
<b>Race</b>			0.323			0.553
White, N (%)	97/122 (79.5)	31/35 (88.6)		64/79 (81.0)	24/27 (88.9)	
Other, N (%)	25/122 (20.5)	4/35 (11.4)		15/79 (19.0)	3/27 (11.1)	
WBC, median (IQR) N, x10 <sup>9</sup> /mCL	10.6 (6.9,17.4) 120	11.2 (6.9,14.8) 44	0.724	10.9 (8.1,17.4) 78	10.6 (6.6,14.9) 34	0.349
WBC ≥ 15, N (%)	41/120 (34.2)	10/44 (22.7)	0.161	28/78 (35.9)	8/34 (23.5)	0.272
Creatinine, median (IQR) N, mg/dL	1.0 (0.8,1.7) 120	1.2 (0.8,1.9) 44	0.407	1.0 (0.8,1.7) 78	1.1 (0.8,1.8) 34	0.358
Creatinine ≥ 1.5, N (%)	38/120 (31.7)	14/44 (31.8)	1.0	22/78 (28.2)	9/34 (26.5)	(1.0)
Albumin, median (IQR) N, mg/dL	2.9 (2.5,3.6) 108	3.4 (3.1,3.8) 21	0.078	2.9 (2.6,3.5) 71	3.4 (3.2,3.8) 16	0.017
ICU admissions, N (%)	16/122 (13.1)	4/44 (9.1)	0.596	8/79 (10.1%)	2/34 (5.9%)	0.721
Colectomy, N (%)	0/122 (0.0)	0/44 (0.0)	N/A	0/79 (0.0%)	0/34 (0.0%)	N/A
Death in 30 days, N (%)	3/122 (2.5)	4/44 (9.1)	0.080	1/79 (1.3%)	1/34 (2.9%)	0.513
Death in 40 days, N (%)	10/122 (8.2)	5/44 (11.4)	0.546	5/79 (6.3%)	2/34 (5.9%)	1.0

027-NAP1-B1, N (%)	15/122 (12.3)	2/44 (4.6)	0.244	15/79 (19.0%)	2/34 (5.9%)	0.090
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CDI, C. difficile infection; NAAT, nucleic acid amplification testing; IQR, Intraquartile range; WBC, white blood count; ICU, Intensive Care Unit. CDI-NAAT and Carrier-NAAT cohorts were defined by positive results on stool NAAT, while CDI-Tox20 and Carrier-Tox20 cohorts included only the subjects that were positive by both NAAT and Single Molecule Array (Simoa; Toxin A+B  $\geq$  20 pg/mL). Laboratory values were from time of enrollment; ICU admission, colectomy, and death rates were assessed within 40 days of enrollment.

<b>Table 2</b>						
<b>Comparison of median toxin concentrations (pg/mL) and NAAT Ct values in CDI and carrier cohorts</b>						
<b>Variable</b>	<b>CDI-NAAT</b>	<b>Carrier-NAAT</b>	<b>p-value</b>	<b>CDI-Tox20</b>	<b>Carrier-Tox20</b>	<b>p-value</b>
Simoa Toxin A concentration, median (IQR), N	50.6 (1.2, 2995.0) 122	60.0 (8.1, 428.9) 44	0.948	874.0 (62.8, 9738.0) 79	129.7 (41.8, 584.1) 34	0.021
Simoa Toxin B concentration, median (IQR), N	89.5 (3.7, 5551.0) 122	42.3 (7.8, 417.0) 44	0.788	1317.0 (99.5, 20229.0) 79	81.7 (26.6, 940.6) 34	0.003
Simoa Toxin A+B concentration, median (IQR), N	197.2 (7.8, 11762.0) 122	137.3 (38.2, 862.4) 44	0.768	4180.7 (215.0, 34916.0) 79	349.6 (123.0, 1342.0) 34	0.004
Xpert ToxinB Ct, median (IQR), N	28.1 (24.9, 32.5) 120	28.6 (25.8, 33.2) 44	0.354	25.8 (23.50, 28.30) 78	27.7 (25.80, 30.7) 34	0.015

CDI, C. difficile infection; NAAT, nucleic acid amplification test; IQR, Intraquartile range. CDI-NAAT and Carrier-NAAT cohorts were defined by positive results on stool NAAT, while CDI-Tox20 and Carrier-Tox20 cohorts included only the subjects that were positive by both NAAT and Single Molecule Array (Simoa; Toxin A+B  $\geq$  20 pg/mL).

**Table 3**

**Median toxin concentration, median Ct value, and % with laxative ingestion in CDI-NAAT and carrier-NAAT cohorts categorized by stool consistency.**

	n	Laxative	Simoa Toxin A	Simoa Toxin B	Simoa Toxin A+B	Ct
<b>CDI</b>	122					
formed	0	n/a	n/a	n/a	n/a	n/a
semi-formed	48	22/48 (46%)	18.8 (0.8, 2436.7)	28.1 (3.0, 7136.3)	113.6 (6.2, 9820.2)	28.8 (25.1, 33.8)
liquid	74	19/74 (26%)	65.6 (1.5, 3670.0)	108.7 (4.10, 5551.0)	258.9 (8.3, 11,762.0)	27.6 (23.6, 31.5)
			p=0.41	p=0.46	p=0.35	p=0.09
<b>Carrier</b>	44					
formed	16	9/16 (56%)	10.7 (1.45, 62.0)	14.45 (7.35, 42.3)	48.8 (8.8, 130.2)	31.7 (26.9, 34.3)
semi-formed	24	16/24 (67%)	129.7 (28.3, 951.1)	87.6 (18.4, 1626.7)	349.6 (88.0, 3054.0)	28.3 (25.7, 31.7)
liquid	4	3/4 (75%)	899 (292.5, 3945.5)	253.5 (5.3, 6275.5)	1152.5 (543.3, 9975.5)	27.1 (25.0, 32.1)
			p<0.01	p=0.06	p<0.01	p=0.36

Laxative data refer to any dose of a relevant oral agent given within the 72h prior to the baseline stool collection. Ct, cycle threshold; CDI, *C. difficile* infection; NAAT, nucleic acid amplification testing. For toxin concentrations and Ct values, data presented are median (IQR).

## Figure Legends

### Figure 1

Dot plots showing distribution of toxin concentrations (measured by Simoa) and Ct values (measured by Xpert NAAT) in symptomatic (CDI-NAAT) vs asymptomatic (Carrier-NAAT) cohorts (defined by positive stool NAAT result). A, Simoa toxin A concentration; B, Simoa toxin B concentration; C, Simoa toxin A+B concentration; D, Xpert cycle threshold (Ct) value. The bottom and top edges of the boxes for each cohort indicate the intra-quartile range, the horizontal line bisecting the box indicates the median value, and the whiskers represent maximum and minimum values. Simoa, single molecule array; CDI, *C. difficile* infection; NAAT, nucleic acid amplification test. P-values for comparison of the respective medians are shown.

### Figure 2

Dot plots showing distribution of toxin concentrations (measured by Simoa) and Ct values (measured by Xpert NAAT) in symptomatic (CDI-Tox20) vs asymptomatic (Carrier-Tox20) cohorts (defined by positive stool NAAT result and stool toxin A+B  $\geq$  20 pg/mL by Simoa). A, Simoa toxin A concentration; B, Simoa toxin B concentration; C, Simoa toxin A+B concentration; D, Xpert cycle threshold (Ct) value. The bottom and top edges of the boxes for each cohort indicate the intra-quartile range, the horizontal line bisecting the box indicates the median value, and the whiskers represent maximum and minimum values. Simoa, single molecule array; CDI, *C. difficile* infection; NAAT, nucleic acid amplification test. P-values for comparison of the respective medians are shown.

### Figure 3

Correlations between toxin concentrations (measured by Simoa) and cycle threshold (Ct) values (measured by Xpert NAAT) and between toxin A and toxin B in the symptomatic cohort defined by positive stool NAAT result (CDI-NAAT). A, Simoa toxin A concentration versus Xpert Ct value; B, Simoa toxin B concentration versus Xpert Ct value; C, Simoa toxin A+B concentration versus Xpert Ct value; D, Simoa toxin A concentration vs Simoa toxin B concentration. Simoa, single molecule array; CDI, *C. difficile* infection, NAAT, nucleic acid amplification testing. Spearman's r values and p-values for each correlation are shown.

### Figure 4

Correlations between toxin concentrations (measured by Simoa) and cycle threshold (Ct) values (measured by Xpert NAAT) and between toxin A and toxin B in the symptomatic cohort defined by positive stool NAAT result and stool toxin A+B concentration  $\geq 20$  pg/mL by Simoa (CDI-Tox20). A, Simoa toxin A concentration versus Xpert Ct value; B, Simoa toxin B concentration versus Xpert Ct value; C, Simoa toxin A+B concentration versus Xpert Ct value; D, Simoa toxin A concentration vs Simoa toxin B concentration. Simoa, single molecule array; CDI, *C. difficile* infection; NAAT, nucleic acid amplification testing. Spearman's r values and p-values for each correlation are shown.

Figure 1.

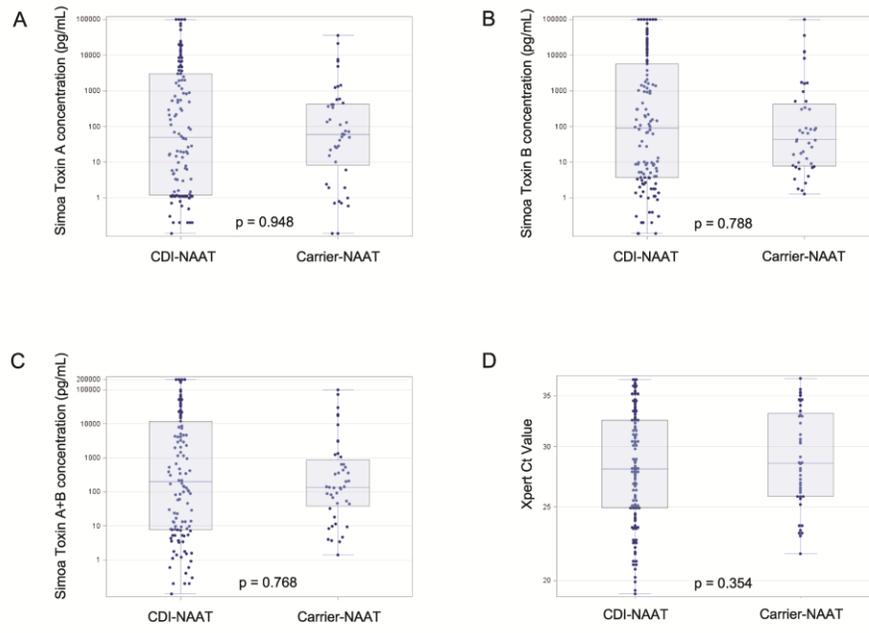


Figure 2.

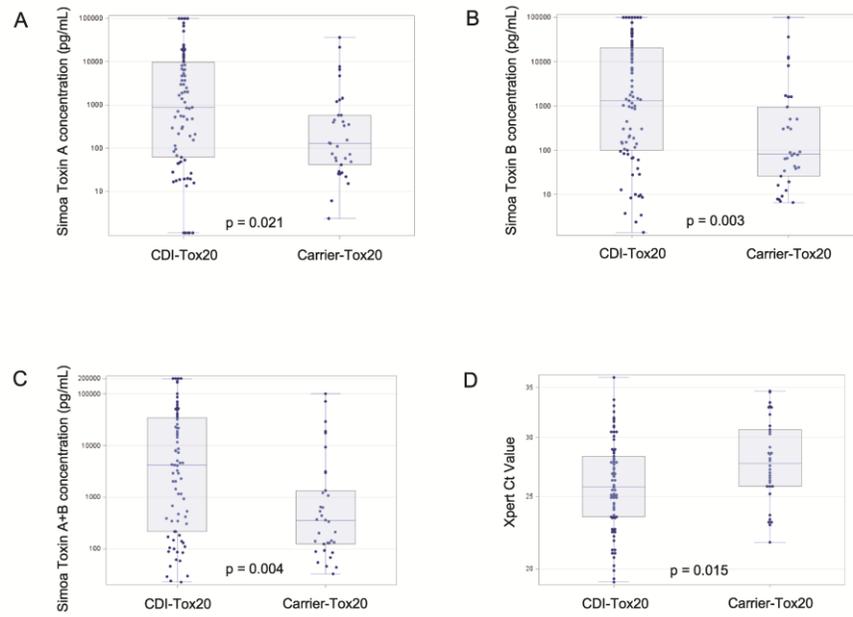


Figure 3.

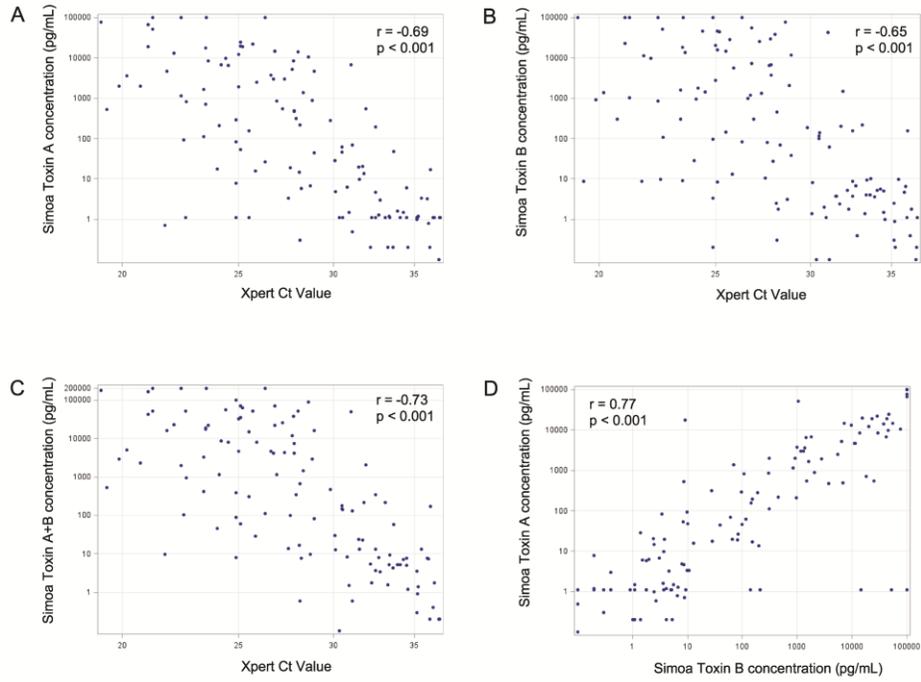


Figure 4.

