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Ultrasensitive and Quantitative Toxin Measurement Correlates With Baseline Severity, Severe Outcomes, and Recurrence Among Hospitalized Patients With *Clostridioides difficile* Infection

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(See the Editorial Commentary by Kraft and Mehta on pages 2150–1.)

Background. Stool toxin concentrations may impact *Clostridioides difficile* infection (CDI) severity and outcomes. We correlated fecal *C difficile* toxin concentrations, measured by an ultrasensitive and quantitative assay, with CDI baseline severity, attributable outcomes, and recurrence.

Methods. We enrolled 615 hospitalized adults (≥18 years) with CDI (acute diarrhea, positive stool nucleic acid amplification testing, and decision to treat). Baseline stool toxin A and B concentrations were measured by single molecule array. Subjects were classified by baseline CDI severity (4 scoring methods) and outcomes within 40 days (death, intensive care unit stay, colectomy, and recurrence).

Results. Among 615 patients (median, 68.0 years), in all scoring systems, subjects with severe baseline disease had higher stool toxin A+B concentrations than those without ($P < .01$). Nineteen subjects (3.1%) had a severe outcome primarily attributed to CDI (group 1). This group had higher median toxin A+B (14 303 pg/mL [interquartile range, 416.0, 141 967]) than subjects in whom CDI only contributed to the outcome (group 2, 163.2 pg/mL [0.0, 8423.3]), subjects with severe outcome unrelated to CDI (group 3, 158.6 pg/mL [0.0, 1795.2]), or no severe outcome (group 4, 209.5 pg/mL [0.0, 8566.3]) ($P = .003$). Group 1 was more likely to have detectable toxin (94.7%) than groups 2–4 (60.5%–66.1%) ($P = .02$). Individuals with recurrence had higher toxin A+B (2266.8 pg/mL [188.8, 29411]) than those without (154.0 pg/mL [0.0, 5864.3]) ($P < .001$) and higher rates of detectable toxin (85.7% versus 64.0%, $P = .004$).

Conclusions. In CDI patients, ultrasensitive stool toxin detection and concentration correlated with severe baseline disease, severe CDI-attributable outcomes, and recurrence, confirming the contribution of toxin quantity to disease presentation and clinical course.

Keywords. *Clostridioides difficile*; toxin concentration; clinical outcomes; recurrence.

Optimal strategies for diagnosis of *Clostridioides difficile* infection (CDI) remain unclear [1]. Exposure to toxinogenic *C difficile* can lead to asymptomatic carriage or to CDI, with clinical presentations ranging from mild diarrhea to severe or even fatal colitis [2, 3]. Recent guidelines stress the importance of combining clinical and laboratory findings to achieve a reliable diagnosis, but clinicians are handicapped by the lack of a single diagnostic gold standard [4]. Currently available approaches include nucleic acid amplification testing (NAAT) for detection of toxin genes, enzyme immunoassay (EIA) detecting *C difficile* toxins A+B, EIA for *C difficile* glutamate dehydrogenase, algorithmic combinations of NAAT and EIAs, or cell cytotoxicity assay (CTA) [1, 2, 4]. The high sensitivity of NAAT is useful for ruling out infection. However, NAAT is insufficiently specific for diagnosis of CDI because it does not distinguish between colonization and infection with the organism [3, 5, 6]. Most recently, the field has reverted toward using toxin EIA for treatment decisions, based on data suggesting that patients

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with toxin-positive stool (versus NAAT-positive, toxin-negative stool) are at higher risk for poor outcomes. However, EIA is analytically insensitive (potentially missing people who may experience CDI-related complications) and provides only binary (positive/negative) results [4–8]. CTA is more sensitive than EIA, but is nonquantitative, subjective, slow, and primarily detects toxin B [2]. Notably, uncertainty remains regarding the relative contribution of *C difficile* toxin A versus toxin B to disease presentation [2].

To address these limitations, we previously developed an ultrasensitive and quantitative toxin immunoassay and have used it to study toxin quantification in diagnosis and outcome prediction [9, 10]. This assay was developed with single molecule array (Simoa) technology, which is based on the high-efficiency capture and labeling of single protein molecules on paramagnetic beads and their detection in arrays of femtoliter-sized wells [9, 11–13]. Our Simoa assay is capable of quantitative measurement of toxin A and toxin B in stool samples at concentrations ranging from picograms per milliliter to high nanograms per milliliter, with an analytical cutoff of ~1 pg/mL and clinical cutoff of 20 pg/mL (for each toxin) in diluted stool samples; our previous studies showed the assay to be more sensitive than CTA for detection of toxin B [9, 10]. We previously found that baseline stool toxin concentrations in patients with CDI (diagnosed by standard clinical and laboratory criteria) overlap substantially with those in asymptomatic carriers [10]. However, when only those with detectable toxin by Simoa were analyzed, the CDI group had significantly higher median toxin concentrations, suggesting that toxin concentration did correlate with presentation and that some of the patients classified as CDI may have been colonized with *C difficile* and had another cause of diarrhea [10]. We then found that specific markers of innate and adaptive immunity in blood and in stool could distinguish CDI from all other groups, suggesting clinical utility for identifying which NAAT and toxin positive patients with diarrhea truly have CDI [14, 15]. Our central hypothesis was that the clinical course of CDI is influenced by the concentrations of toxins A and B in the colon, and thus that accurate and quantitative stool toxin measurement can improve the diagnosis of CDI, aid prediction of disease outcomes, and guide management. In this study, we have used the Simoa assay to assess the contribution of toxin quantity to disease presentation and clinical course in patients with CDI, investigating the impact of toxin concentration on disease severity at diagnosis, severe CDI-attributable outcomes, and CDI recurrence.

METHODS

Study Population, Clinical Data Collection, and Attribution

Eligible inpatients at Beth Israel Deaconess Medical Center (BIDMC; Boston, Massachusetts) and Texas Medical Center Hospitals (TMC; Houston, Texas) were prospectively enrolled between June 22, 2016, and July 12, 2019, under protocols approved by the institutional review boards at each institution. Subjects were ≥18 years old with positive stool *C difficile* NAAT result, initiating CDI therapy, and had acute diarrhea (definition in Supplementary Methods). The diagnostic clinical stool sample (submitted for routine *C difficile* testing) was captured as a discarded sample.

The study team scored patients using 4 separate CDI severity scoring guidelines (Infectious Diseases Society of America [IDSA], European Society of Clinical Microbiology and Infectious Diseases [ESCMID], Zar et al, and Belmares et al) as previously described [16–20] and scored severe outcomes (intensive care unit [ICU] admission, colectomy, or death) and recurrence within 40 days of study enrollment (severe outcomes within 30 days were also recorded). Details of diarrhea assessment, subject exclusion, clinical data collection, and outcome definitions/attributions are in Supplementary Methods.

Sample Processing and Analysis

Eligible stool samples were captured, kept refrigerated, and aliquoted and frozen at −80°C within 72 hours of stool sample collection. For stool samples clinically tested by the Xpert C. difficile/Epi assay (Supplementary Methods), cycle thresholds (Ct values) for the *C difficile tcdB* gene were recorded; all remaining BIDMC study stool samples and 9/237 (3.8%) of TMC samples also were tested with the Xpert assay to capture Ct value data. Toxin A and B measurements were performed using Simoa assays at bioMérieux (Lyon, France), as previously described [10, 14]. Any toxin A or toxin B measurements below the clinical cutoff of 20 pg/mL were converted to 0 for analysis. A positive toxin result was therefore defined as either toxin A or B ≥20 pg/mL.

Statistical methods and sample size/power calculations are detailed in Supplementary Methods.

RESULTS

There were 1625 subjects assessed for enrollment in the study (Supplementary Figure 1). After exclusions, we enrolled 615 subjects, whose demographic and clinical characteristics are shown in Table 1. A total of 380/615 enrolled at BIDMC and 237/615 at TMC. Subjects had a median age of 68 years, and 53.3% were female. Most subjects (76.4%) were white. Laboratory markers of severity, including white blood cell ≥15 K/μL, creatinine ≥1.5 g/dL, and albumin <3 mg/dL were noted in 32.1%, 37.3%, and 49.2% of subjects, respectively. By Simoa, 363/615 (59%) had detectable toxin A, 381/615 (62%) detectable toxin B, and 406 (66.0%) detectable toxin A or B. A total of 159/615 (25.9%) subjects had detectable toxin that was below 1000 pg/mL (the estimated sensitivity of EIA). One hundred and thirty-one subjects (21.3%) had a severe outcome (ICU admission [n = 108], colectomy [n = 7], or death [n = 43])

Table 1. Demographics, Baseline Laboratory Features, and Clinical Outcomes for Study Participants With CDI

Baseline Characteristics of Enrolled Subjects (n = 615)	
Age (y), median (IQR)	68.0 (55.0, 77.0)
Sex	
Female	329 (53.3%)
Male	286 (46.5%)
Race	
White	447 (76.4%)
Other	138 (23.6%)
Ethnicity	
Hispanic	53 (8.6%)
Non-Hispanic	562 (91.4%)
Laboratory results	
WBC K/μL, median (IQR)	11.7 (7.2, 16.6)
WBC ≥15K/μL (n, %)	196 (32.1%)
Creatinine g/dL, median (IQR)	1.1 (0.8, 2.0)
Creatinine ≥1.5 g/dL (n, %)	228 (37.3%)
Albumin mg/dL, median (IQR)	2.9 (2.5, 3.5)
Albumin <3 mg/dL (n, %)	281(49.2%)
027-NAP1-BI (n, %)	50 (13.0%)
Outcomes[a]	
ICU admission	108 (17.6%)
Colectomy	7 (1.1%)
Death	43 (7.0%)
Any severe outcome[b]	131 (21.3%)
LOS (days)	5.0 (2.0, 9.0)

Note: Data are n (%) unless otherwise indicated. Race information available for 585 subjects; ethnicity information available for 615 subjects; WBC and creatinine available for 611 subjects; albumin available for 571 subjects; NAP-1 information (Xpert) available for 384 subjects; LOS available for 615 subjects. ICU admission, colectomy, death, and CDI recurrence were assessed within 40 days of enrollment.

Abbreviations: CDI, *Clostridioides difficile* infection; IQR, interquartile range; ICU, intensive care unit; LOS, length of stay; WBC, white blood cell.

[a]There were 105 ICU admissions (17.1%), 6 colectomies (1.0%), and 34 deaths (5.5%) within 30 days.

[b]Any severe outcome included ICU admission, colectomy, or death within 40 days.

within 40 days. (Within 30 days, there were 105 ICU admissions, 6 colectomies, and 34 deaths.)

We explored the association between stool toxin concentration and baseline disease severity using 4 CDI severity measures (IDSA-Society for Healthcare Epidemiology [SHEA], Zar, Belmares, and ESCMID) (Table 2) [4, 17–19]. Correlation was strongest with disease severity as assessed by ESCMID and Zar criteria, but also highly significant for the 2 other scoring systems (IDSA-SHEA and Belmares). Notably, despite observing significantly higher concentrations of stool toxins A, B, and A+B in the severe CDI groups by all 4 criteria used, no significant differences in Xpert Ct values were observed between the severe and not severe groups (Table 2). For the ESCMID and Zar scoring systems, patients with severe disease were significantly more likely to have detectable toxin by Simoa than those without severe disease (Table 3).

Next, we explored the association between stool toxin levels and severe outcomes (death, ICU stay, or colectomy within 40 days) according to CDI attribution status. Patients were characterized as having 1 of 4 attributions: severe outcome that was primarily attributable to CDI (group 1, n = 19 [3.1%]), severe outcome where CDI contributed to the outcome (group 2, n = 43 [7.0%]), severe outcome not attributable to CDI (group 3, n = 69 [11.2%]), or no severe outcome (group 4, n = 484 [78.7%]) (Figure 1). This analysis showed a highly statistically significant association between the concentration of stool toxins A and B and primarily attributable severe CDI outcomes (Figure 1A-C), with an approximately 100-fold difference in median concentration of stool toxins A+B between patients with primarily attributed severe outcomes (group 1) and groups 2–4. In contrast, there was no difference in median Ct values between these groups (Figure 1D). The same findings were observed when outcomes at 30 days (rather than 40) were assessed (data not shown). A statistically significant difference was also seen in the proportion of individuals in each group who had detectable toxin by Simoa, with 94.7% toxin positive in group 1 versus 60.5% in group 2, 60.9% in group 3, and 66.1% in group 4 ($P = .230$ for 4-way comparison; $P = .010$ for comparison between group 1 and group 4, Table 3).

We also examined the association between stool toxin concentration and CDI recurrence within 40 days. Subjects who

Table 2. Association of Baseline Stool Toxin Concentration With Baseline CDI Severity

Severity Score	Not Severe	Severe	*P* Value
IDSA-SHEA	n = 278 median (IQR)	n = 337 median (IQR)	
Simoa toxin A	40.1 (0.0, 1152.5)	120.0 (0.0, 4262.5)	.006
Simoa toxin B	36.7 (0.0, 1787.2)	295.5 (0.0, 10437)	<.001
Simoa toxin A+B	114.2 (0.0, 3612.9)	576.3 (0.0, 17029)	.002
Xpert toxin B Ct[a]	28.0 (23.6, 32.5)	27.2 (23.6, 31.1)	.157
Zar	n = 347 median (IQR)	n = 268 median (IQR)	
Simoa toxin A	37.2 (0.0, 1152.5)	206.8 (0.0, 5823.7)	<.001
Simoa toxin B	40.2 (0.0, 2070.0)	374.5 (0.0, 16919)	<.001
Simoa toxin A+B	119.0 (0.0, 4180.7)	728.6 (0.0, 22236)	<.001
Xpert toxin B Ct[a]	27.4 (23.4, 32.2)	27.4 (24.1, 30.8)	.744
Belmares	n = 540 median (IQR)	n = 75 median (IQR)	
Simoa toxin A	71.0 (0.0, 1804.4)	120.6 (0.0, 14373)	.033
Simoa toxin B	83.5 (0.0, 3074.2)	506.8 (0.0, 41953)	.003
Simoa toxin A+B	204.4 (0.0, 5864.3)	711.0 (0.0, 62253)	.009
Xpert toxin B Ct[a]	27.5 (23.5, 31.7)	27.3 (24.3, 31.4)	.977
ESCMID	n = 264 median (IQR)	n = 351 median (IQR)	
Simoa toxin A	0.0 (0.0, 1078.2)	148.2 (0.0, 4931.0)	<.001
Simoa toxin B	20.7 (0.0, 1209.5)	304.1 (0.0, 9975.8)	<.001
Simoa toxin A+B	55.1 (0.0, 3053.5)	676.5 (0.0, 17612)	<.001
Xpert toxin B Ct[a]	28.3 (24.0, 33.1)	27.2 (23.5, 31.1)	.059

Note: All values are in picograms per milliliter unless otherwise indicated.

Abbreviations: CDI, *Clostridioides difficile* infection; Ct, cycle threshold; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; IDSA, Infectious Diseases Society of America; IQR, interquartile range; SHEA, Society for Healthcare Epidemiology; Simoa, single molecule array; Simoa toxin A, *C difficile* toxin A concentration by Simoa assay; Simoa toxin B, *C difficile* toxin B concentration by Simoa assay; Simoa toxin A+B, the sum of the concentrations of *C difficile* toxins A and B by Simoa assay.

[a]A total of 384 subjects underwent testing by Xpert. By severity score, the proportion of severe and not severe subjects tested by Xpert were as follows: IDSA (163 not severe/221 severe), Zar (219 not severe/165 severe), Belmares (328 not severe/56 severe), and ESCMID (143 not severe/241 severe).

Table 3. Proportion of Subjects With Detectable Toxin by Simoa by Baseline Severity, Severe Outcomes, and Recurrence

	Toxin A or B ≥20 pg/mL (Detectable Toxin)		
	No Detectable Toxin	Detectable Toxin	*P* Value
Baseline severity score	n, %	n, %	
IDSA-SHEA			.147
Not severe (n = 278)	103 (37.1%)	175 (62.9%)	
Severe (n = 337)	106 (31.5%)	231 (68.5%)	
Zar			.026
Not severe (n = 347)	131 (37.8%)	216 (62.2%)	
Severe (n = 268)	78 (29.1%)	190 (70.9%)	
Belmares			.118
Not severe (n = 540)	190 (35.2%)	350 (64.8%)	
Severe (n = 75)	19 (25.3%)	56 (74.7%)	
ESCMID			<.001
Not severe (n = 264)	113 (42.8%)	151 (57.2%)	
Severe (n = 351)	96 (27.4%)	255 (72.6%)	
Severe outcome			.010
No severe outcome (n = 484) (group 4)	164 (33.9%)	320 (66.1%)	
Severe outcome primarily attributed to CDI (n = 19) (group 1)	1 (5.3%)	18 (94.7%)	
Recurrence			.004
No recurrence (n = 500)	180 (36.0%)	320 (64.0%)	
Recurrence (n = 42)	6 (14.3%)	36 (85.7%)	

Note: 20 pg/mL is the clinical cutoff for quantitative measurement of toxin A and toxin B by Simoa. For the severe outcome category, not shown here are the subjects with a severe outcome where CDI contributed (43/615) (group 2) and those with a severe outcome that was not related to CDI (69/615) (group 3). Patients were excluded from analysis of recurrence if they remained on CDI treatment throughout the 40-day follow-up period. There were 542 subjects from the initial population of 615 subjects included in the recurrence analysis.
Abbreviations: CDI, *Clostridioides difficile* infection; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; IDSA, Infectious Diseases Society of America; SHEA, Society for Healthcare Epidemiology; Simoa, single molecule array.

received CDI therapy continuously throughout the 40-day follow-up period were excluded from the analysis, leaving 542 evaluable subjects; 42/542 (7.7%) had recurrence and 500/542 (92.3%) did not. Subjects with recurrence had significantly higher median baseline stool concentrations of toxins A, B, and A+B compared with subjects who did not have recurrence, whereas median Xpert Ct values did not differ between these 2 groups (Figure 2). Consistent with this finding, the proportion of those with recurrence who were toxin positive at baseline (85.7%) was significantly higher than for those without recurrence (64.0%) ($P = .004$) (Table 3).

DISCUSSION

The chronic confusion around CDI diagnosis suggests that approaches to diagnosis of this disease need not only clarification, but potentially redefinition. Currently, clinicians are uncertain of how to integrate test results with clinical findings, particularly given confusion about which results in multistep testing algorithms should be used for treatment decisions versus administrative reporting of nosocomial infections [21]. Although current guidelines suggest that clinical diarrhea and a "positive test result" are sufficient to diagnose CDI, it is not clear which of the clinically available imperfect test choices provides the best diagnostic information.

This study was motivated by the recognition that multiple preliminary studies had suggested, but never proven, a correlation between the amount of toxin in stool and disease severity [6, 22–25]. Most of these studies were small, and correlations indirect. Until our development of the Simoa assay, there had never been a tool with which to sensitively detect and separately quantify both toxins A and B in stool over the necessary concentration ranges, and thus with which to directly correlate toxin quantities with clinical course. Although the data appeared to indicate that detection of toxin, rather than of bacteria capable of producing that toxin, should be the cornerstone of accurate diagnosis of CDI, we believed it critical to directly test the hypothesis that the clinical course of CDI is influenced by the concentrations of toxins A and B in the colon, and thus that accurate and quantitative stool toxin measurement can improve the diagnosis of CDI, aid prediction of disease outcomes, and guide management. Since initiation of our study, a small number of studies have been published regarding the relationship between CDI severity and toxin concentration; although supportive of our hypothesis, the studies have been small, had insufficient clinical correlation, or used nonquantitative methods [7, 26, 27]. In short, before this work, no group has convincingly demonstrated that stool toxin quantification has additional diagnostic or prognostic value.

Our data from this study clearly demonstrate that, in our population of patients with CDI, stool toxin concentrations are significantly higher in patients with severe CDI at diagnosis. Furthermore, higher stool toxin concentrations at diagnosis predict severe CDI-attributable outcomes and CDI recurrence, confirming the contribution of toxin quantity to disease

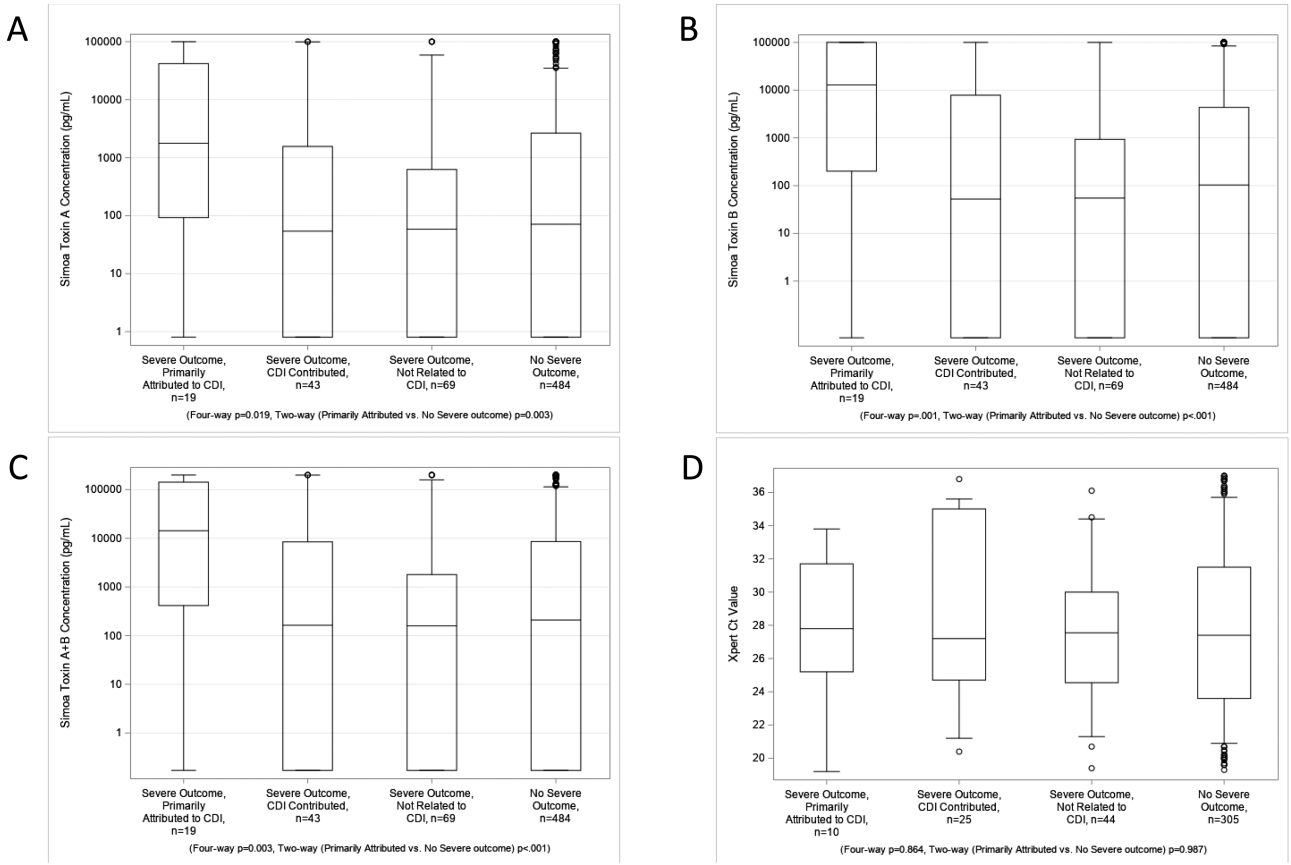

Figure 1. Dot plots showing distribution of toxin concentrations (measured by Simoa) and Ct values (measured by Xpert NAAT) in patients with a severe outcome primarily-attributable to CDI, those in whom CDI contributed to a severe outcome, those who had a severe outcome unrelated to CDI, and those without a severe outcome. (*A*) Simoa toxin A concentration. (*B*) Simoa toxin B concentration. (*C*) Simoa toxin A+B concentration. (*D*) Xpert Ct value. The bottom and top edges of the boxes for each cohort indicate the interquartile range, the horizontal line bisecting the box indicates the median value, and the whiskers represent 5% and 95% values; outliers are represented by circles. *P* values for comparison of the respective medians (4-way, all 4 groups compared; 2-way, primarily attributable severe outcome compared with no severe outcome) are shown. Abbreviations: CDI, *Clostridioides difficile* infection; Ct, cycle threshold; NAAT, nucleic acid amplification test; Simoa, single molecule array.

presentation and clinical course. Notably, Ct values did not show the same correlations, in contrast to 2 other studies that have noted an association between low Ct values and poor outcomes [28, 29]. Notably, both of those studies bifurcated Ct values as being above or below a specified cutoff (<23.5 and ≤25, respectively), as opposed to analyzing Ct value as a continuous variable as performed in this study [28, 29]. We have previously shown that toxin concentrations and Ct values roughly correlate [10], and thus would expect categorically low Ct values to be a reasonable proxy for categorically high toxin concentrations [28–30].

We previously showed that stool toxin concentrations in patients with CDI (either NAAT or toxin positive, plus new-onset diarrhea) overlap substantially with those in symptomless carriers [10]. However, in that study, when we evaluated only subjects with detectable toxin (defined as stool toxin A+B ≥20 pg/mL by Simoa), we observed that the CDI cohort had significantly higher median toxin concentrations than the carrier cohort, suggesting that toxin concentration in fact contributed to clinical presentation. We concluded that stool toxin A and toxin 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, as above, our results also demonstrated that when considered as a group, toxin concentrations were significantly higher in toxin-positive CDI patients than in toxin-positive carriers [10], adding strength to the argument that detection of toxin is more clinically relevant than detection of the toxin B gene. In this study, we also found overlap in toxin concentration distributions between individuals with and without severe disease and/or severe outcomes. This is not surprising given the likelihood that toxin concentration is but one contributor to CDI severity and clinical course; other contributors include features such as immune status, age, and comorbidities. Nonetheless, toxin concentrations were clearly associated with more severe baseline disease and outcomes in this study, supporting the role of toxin concentration in disease expression.

Taking all of these data together, we infer that in an individual patient, the lack of detectable toxin by Simoa makes it unlikely

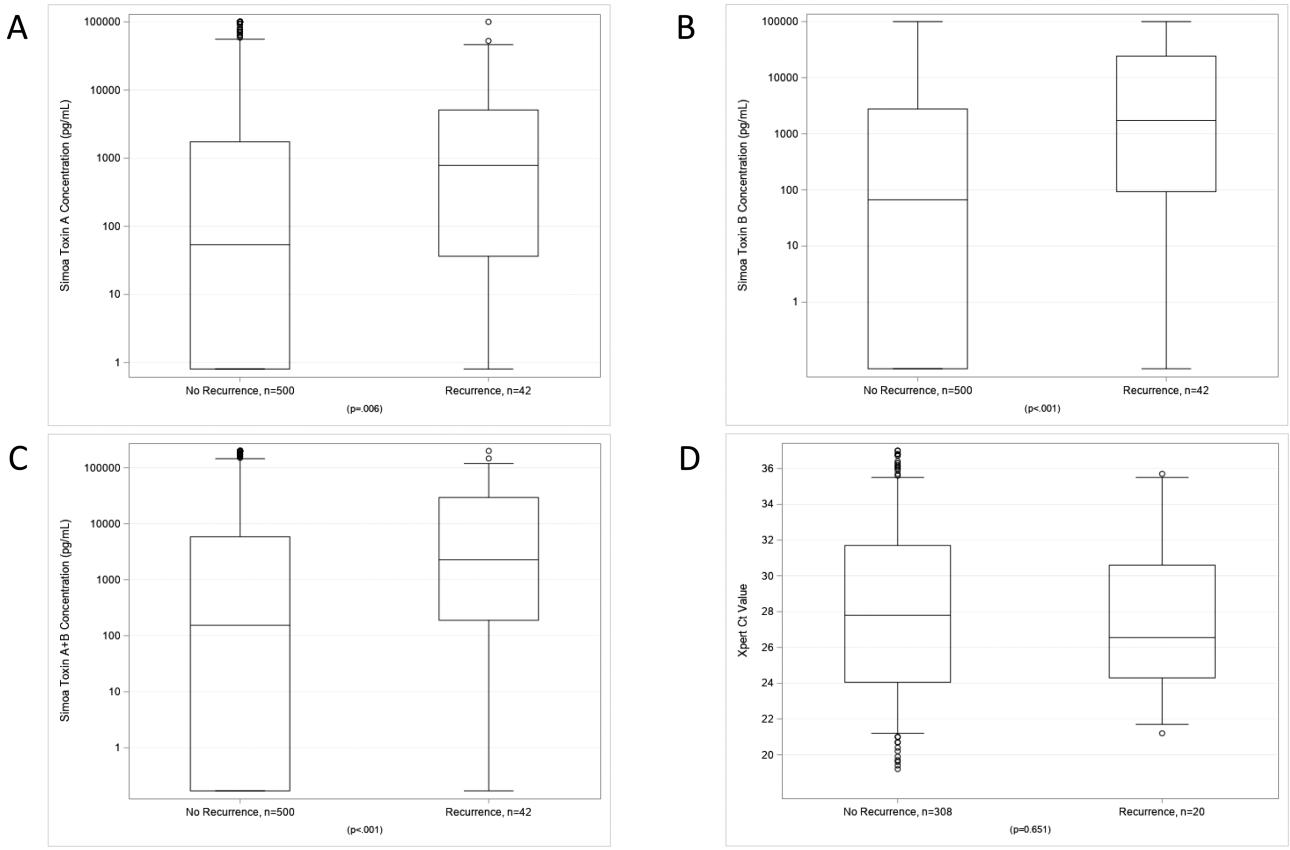

Figure 2. Dot plots showing distribution of toxin concentrations (measured by Simoa) and Ct values (measured by Xpert NAAT) in patients without CDI recurrence and with CDI recurrence within 40 days. (*A*) Simoa toxin A concentration. (*B*) Simoa toxin B concentration. (*C*) Simoa toxin A+B concentration. (*D*) Xpert Ct value. The bottom and top edges of the boxes for each cohort indicate the interquartile range, the horizontal line bisecting the box indicates the median value, and the whiskers represent 5% and 95% values; outliers are represented by circles. *P* values for comparison of the respective medians are shown. Abbreviations: CDI, *Clostridioides difficile* infection; Ct, cycle threshold; NAAT, nucleic acid amplification test; Simoa, single molecule array.

that the patient has true CDI, but neither stool toxin concentration, nor NAAT Ct value, can reliably distinguish true CDI from a symptomatic, colonized patient whose diarrhea has another cause. Given that 34% of our NAAT-positive study cohort did not have detectable stool toxin by Simoa, we acknowledge that this proportion of our study patients may have actually had a separate cause of their diarrhea other than CDI. However, our findings that higher baseline toxin concentrations correlate with more severe baseline disease and outcomes, and that those with more severe disease and adverse outcomes were significantly more likely to be toxin positive by Simoa, suggest that ultrasensitive detection and quantification of toxins has value for both diagnosis and outcome prediction.

Here, we demonstrate a strong association between baseline stool toxin concentrations and subsequent risk of recurrent CDI (rCDI). One recent study suggested that toxin-positive (versus NAAT-positive, toxin-negative) patients were more likely to have recurrence, but the toxin assay used was an EIA with nonquantitative results [7]. Prior work has suggested clinical variables such as age, severity of illness by the Horn score, and concomitant non-CDI antibiotic use are associated with the strongest risk of rCDI [31]. It is possible that the explanation for the association between toxin and recurrence might be our observation that patients with higher stool toxin concentrations had higher severity of disease at baseline, thus providing a risk factor for rCDI. The present study was limited in our ability to fully explore all of the clinical variables associated with rCDI risk and it was not designed to capture all concomitant non-CDI antibiotics and underlying conditions that may play a role in rCDI. Future studies are planned to evaluate these associations further.

One advantage of this study was the ability to measure the stool concentrations of toxins A and B independently. This allowed us to show that both toxin A and toxin B concentrations were associated with severe disease, though toxin B concentrations were slightly more highly predictive overall. Our prior work has demonstrated that there are toxin A-predominant strains [32], and toxin A−/B+ strains are well-known to cause clinical disease [33, 34], suggesting that detection and quantification of both toxins A and B is optimal.

Our study had the following limitations. First, we fully acknowledge that our definition of CDI may have led to inclusion

of some subjects who in fact did not have CDI, and that attributing outcomes as CDI-related or CDI-unrelated is subjective (though informed by expertise and experience). Our inclusion criteria required that patients be positive by NAAT and have diarrhea meeting IDSA-SHEA criteria [4]. As noted previously, 34% of the CDI subjects in our study were NAAT+/Simoa−, which either means that they do not have CDI or they do have CDI, but have toxin A and toxin B concentrations both below 20 pg/mL. However, of note, 18/19 (95%) of the individuals we identified as having CDI-attributable severe outcomes had detectable toxin by Simoa. A limitation of our study design is that we did not collect complete data on alternative causes of diarrhea. Nonetheless, we do not think that this undermines our findings comparing those with severe disease and severe outcomes to those without. We note that our study included subjects with empiric pretreatment (up to 48 hours) before stool sample collection, which may have depressed measured toxin concentrations in some patients. However, this does not detract from our finding that toxin concentrations were higher in those with more severe disease, particularly because those with severe disease were actually more likely to be given empiric pretreatment (data not shown). We note that the frequency of 40-day recurrences was relatively low in this cohort (7.7%) compared with other studies that have estimated recurrence rates between 15% and 35% [35]. This may be a reflection of a short window in which to recur because most subjects received at least 10 days of CDI therapy, and many patients received prolonged treatment courses (data not shown), leaving a smaller window in which to capture recurrent episodes. Although every effort was made to follow patients for recurrent CDI episodes, we may have missed cases if the subject recurred after discharge or if the subject did not return to our medical center for follow-up care. The association of recurrence and toxin concentration will be analyzed in more detail in future dedicated studies. Finally, the current lack of access to commercially available ultrasensitive and quantitative toxin assays for clinical use is a limitation to the field; we hope and anticipate that our work may stimulate commercial development of such assays.

In conclusion, our cumulative work to date suggests that ultrasensitive toxin detection and quantification by Simoa is useful in ruling out CDI, identifying severe CDI, and predicting both severe clinical outcomes and recurrence. Our data reinforce the conclusion that accurate diagnosis of CDI requires both a positive *C difficile* stool test and rigorous confirmation of CDI symptoms. Future prospective studies can now be done to evaluate the performance of the stool Simoa assay, possibly in combination with novel biomarkers to more specifically identify CDI-associated colitis, as a single-step diagnostic approach, comparing its diagnostic accuracy to current laboratory tests and algorithms and evaluating its added value for identification of severe disease and outcome prediction [14, 15, 36, 37]. Our ultimate goal is to define a novel, highly accurate, single-step diagnostic strategy for CDI that can be easily deployed by clinicians without specific expertise in CDI diagnosis. We suspect that ultrasensitive toxin detection and quantification in stool, combined with measurement of innate and adaptive immune biomarkers to augment specificity, will provide optimal diagnostic accuracy and outcome prediction in patients with suspected CDI, offering the possibility to improve and perhaps transform CDI diagnosis.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. C. P. K. and N. R. P. conceived and designed the study. All members of the study group acquired the data. C. D. A., C. P. K., K. W. G., A. J. G.-L., D. W., K. D., J. V.-G., X. C., M. M., A. B., and N. R. P. analyzed and interpreted the data. C. D. A., C. P. K., and N. R. P. drafted the manuscript. Critical revisions to the manuscript were made by all members of the study group. C. P. K. and N. R. P. obtained the funds for the study. C. D. A., C. P. K., D. W., K. D., and N.R.P. verified all data. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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