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Role of Fecal *Clostridium difficile* Load in Discrepancies Between Toxin Tests and PCR: Is Quantitation the Next Step in *C. difficile* Testing?

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Abstract

Purpose—Direct tests for *Clostridium difficile* are 30–50% more sensitive than tests for *C. difficile* toxins but the reasons for this discrepancy are incompletely understood. In addition to toxin degradation and strain differences, we hypothesized that *C. difficile* concentration could be important in determining whether toxins are detected in fecal samples.

Methods—We performed standard curves on an FDA-approved real-time PCR test for the *C. difficile tcdB* gene (Xpert *C. difficile*/Epi, Cepheid) during a prospective comparison of a toxin immunoassay (Meridian Premier), PCR and toxigenic culture. Immunoassay-negative, PCR-positive samples were retested with a cell cytotoxin assay (TechLab).

Results—Among 107 PCR-positive samples, 46 (43.0%) had toxins detected by immunoassay and an additional 18 (16.8%) had toxin detected by the cytotoxin assay yielding 64 (59.8%) toxin-positive and 43 (40.2%) toxin-negative samples. Overall, toxin-negative samples with *C. difficile* had 10¹–10⁴ fewer DNA copies than toxin-positive samples and most discrepancies between toxin tests and PCR were associated with a significant difference in *C. difficile* quantity. 95% of toxin-positive samples had 4.1 log₁₀ *C. difficile tcdB* DNA copies/mL. 52% of immunoassay-negative samples and 70% of immunoassay and cytotoxin negative samples had <4.1 log₁₀ *C. difficile tcdB* DNA copies/mL.

Conclusions—These findings suggest that fecal *C. difficile* concentration is a major determinant of toxin detection and *C. difficile* quantitation may add to the diagnostic value of existing test methods. Future studies are needed to validate the utility of quantitation and determine the significance of low concentrations of *C. difficile* in the absence of detectable toxin.

Keywords

Clostridium difficile; toxin; PCR; quantitation; diarrhea

INTRODUCTION

C. difficile infection (CDI) is due to an overgrowth of *C. difficile* with production of toxins that typically occurs in the setting of prior antibiotics. Traditionally, diagnosis of CDI has relied on detection of *C. difficile* toxins in feces because toxins are essential mediators of disease and may help distinguish patients with CDI from carriers [1]. More recently, concern that patients with CDI were missed by toxin tests has prompted clinical laboratories to switch to methods that detect *C. difficile* directly, such as real-time PCR [2–4]. These tests are 30–50% more sensitive for *C. difficile* than toxin tests but the reasons for this difference are incompletely understood [3, 5, 6]. Pre-analytic toxin degradation and antigenic variation between *C. difficile* strains account for some of this difference but the impact of the *C. difficile* concentration on detection by toxin tests is not well characterized [6, 7]. We hypothesized that toxin-negative results could be due to a lower *C. difficile* concentration in the sample and that *C. difficile* quantitation could explain many of the discrepancies between toxin tests and culture or PCR. To evaluate this, we performed standard curves with an FDA-approved real-time PCR test for *C. difficile* to determine the *C. difficile* fecal load in samples that tested positive or negative by a toxin A and B immunoassay. Toxin immunoassay-negative, PCR-positive samples were reevaluated by a cell cytotoxin assay. Using this approach, we examined the relationship between the *C. difficile* fecal load and toxin detection by immunoassay and cell cytotoxin assay for 107 PCR-positive samples including BI/NAP1/027 strains and non-BI/NAP1/027 *C. difficile* strains.

MATERIALS AND METHODS

Consecutive diarrheal stool samples from adult inpatients submitted for *C. difficile* testing 72 hours after admission from January to October, 2011 were tested by a toxin immunoassay (Premier *C. difficile* Toxins A&B, Meridian Bioscience), real-time PCR for the *C. difficile* toxin B gene (*tcdB*) and two markers of the BI/NAP1/027 strain (Xpert *C. difficile*/Epi, Cepheid) and alcohol-shock anaerobic culture (CCFA-ST, Remel). The current study was limited to PCR-positive samples from unique patients. The toxin immunoassay, PCR and anaerobic stool culture were performed on fresh stool samples within 24, 36 and 48 hours of collection, respectively. All samples were transported on ice and kept refrigerated (2–8° C) prior to testing to minimize pre-analytic degradation. Toxin immunoassay-negative, PCR-positive samples were retested by a cell culture cytotoxin assay (Wampole *C. difficile* Tox-B, TechLab) with MHRF cells (Diagnostic Hybrids) from frozen aliquots when available (n=55/61, 90.2% of samples). All tests were performed according to the manufacturer's package insert. For culture, 0.5 mL of stool was treated with an equivalent volume of 70% EtOH for 10 minutes and plated to pre-reduced CCFA-ST media. Cultures were evaluated for 3 days and suspicious colonies were identified by a combination of colony and gram stain morphology, odor, production of L-proline-aminopeptidase and fluorescence under long-range UV light. In-vitro toxin production of *C. difficile* isolates was confirmed using broth from 48 hour culture in chopped meat broth (Remel) [8]. PCR cartridges were inoculated in a class II biological safety cabinet located away from the PCR instrument. Standard curves were determined for each real-time PCR lot to allow the initial concentration of *C. difficile tcdB* DNA in feces (copies/mL) to be calculated from the PCR cycle number at the end point of detection. Briefly, 100 µL of serial 10-fold dilutions of a 0.5 McFarland suspension from a 24-hour culture of *C. difficile* ATCC 43255 were plated to Brucella agar in triplicate and also frozen. The average colony count was used to determine the concentration of *C. difficile* in each dilution and 100 µL of each dilution was tested in duplicate by real-time PCR. Since the real-time PCR method we used utilizes a swab to transfer feces from the clinical sample into lysis buffer, the volume of feces per PCR test was assumed to be 100 µL for these calculations based on an average stool weight of 0.095g for five replicate swabs from a typical loose stool sample. The

reproducibility of quantitation was evaluated by replicate testing (5x) of frozen stool aliquots from 3 samples with high, medium and low concentrations of *C. difficile* DNA at the time of initial testing. Samples were designated as positive or negative for the BI/NAP1/027 strain based on the real-time PCR results for the binary toxin gene and *tcdC* gene deletion using the default software settings. In addition, 77 of 100 (77%) samples with *C. difficile* recovered by culture had independent confirmation of the fecal real-time PCR 027/non-027 designation by direct PCR ribotyping of the isolate at the time of this manuscript.

The distributions of $\log_{10}$ *C. difficile* DNA/mL fecal concentrations were compared between groups using the Wilcoxon rank-sum test. The Chi-squared test or Fisher's exact test were used for frequency comparisons as appropriate. Receiver operating characteristic (ROC) curve analysis was done in STATA, version 10.1.

RESULTS

During the study period, 107 unique inpatients had samples that were positive for *C. difficile tcdB* DNA by PCR. Of these, 46 (43.0%) had toxins detected by EIA and an additional 18 (16.8%) had toxins detected by cell culture cytotoxicity yielding 64 (59.8%) samples with toxins detected (toxin-positive) and 43 (40.2%) samples with no toxins detected (toxin-negative). By quantitative PCR, toxin-negative samples had 10^1 – 10^4 (10–10,000) times less *C. difficile* DNA than toxin-positive samples with a median concentration of 3.68 $\log_{10}$ *C. difficile tcdB* copies/mL [95% CI 3.45, 4.05] in toxin-negative samples and 5.91 $\log_{10}$ copies/mL [95% CI 5.56, 6.00] in toxin-positive samples (Figure 1B, $P < 0.0001$). By culture, toxigenic *C. difficile* was recovered from 100/107 (93.5%) samples including 63/64 (98.4%) toxin-positive samples and 37/43 (86.1%) toxin-negative samples. The median *C. difficile* DNA concentration in culture-negative, PCR-positive samples was 2.69 $\log_{10}$ copies/mL [95% CI 2.52, 3.72] feces. The ROC curve for $\log_{10}$ *C. difficile tcdB* DNA copy number versus the combined toxin result showed an area under the curve of 0.921 [95% CI 0.870, 0.973] suggesting that *C. difficile* quantitation was sensitive and specific for toxin detection in our population (Figure 2). For example, an arbitrary high sensitivity cutoff of 4.1 $\log_{10}$ DNA copies/mL correctly classified 95% ($n=61/64$) of toxin-positive samples (sensitivity) and 70% ($n=30/43$) of toxin-negative samples (specificity) (Figure 1B and Figure 2). With the same cutoff, the positive predictive value of 4.1 $\log_{10}$ DNA copies/mL for detectable toxins in the clinical stool sample was 82.4% and the negative predictive value of <4.1 $\log_{10}$ DNA copies/mL was 90.9%. The sample distribution was more mixed with the toxin immunoassay alone (i.e., more overlap between immunoassay positive and negative samples) with 48% ($n=29/61$) of negative samples having 4.1 $\log_{10}$ DNA copies/mL and 52% having <4.1 $\log_{10}$ DNA copies/mL (Figure 1A).

By strain type, stool samples with the hypervirulent BI/NAP1/027 *C. difficile* strain were 3 times more likely to be toxin-positive ($n=21/27$, 77.8%) than samples with a non-027 strain ($n=43/80$, 53.8%; O.R. = 3.0 [95% CI 1.1, 8.3]; $P=0.028$) and toxin-negative, BI/NAP1/027 samples had less *C. difficile*/mL than toxin-negative, non-027 samples (2.88 $\log_{10}$ *tcdB* DNA copies/mL vs. 3.74 $\log_{10}$ *tcdB* DNA copies/mL, $P=0.008$) (Figure 1B). Of the 77 isolates with PCR ribotype results, 12/12 (100%) isolates from samples reported “presumptive 027” by real-time PCR were confirmed to be a ribotype 027 strain and 65/65 isolates from non-027 samples either matched a non-027 ribotype (total, $n=46$; 001, $n=14$; 014, $n=4$; 056, $n=3$; 078, $n=1$; 087, $n=8$; 106, $n=4$; other miscellaneous ribotypes, $n=12$) or failed to match a ribotype in the database ($n=19$).

To evaluate the reproducibility of *C. difficile* quantitation from fecal samples using our methods, we tested three stool samples (high, medium, and low *C. difficile* concentrations) in replicate, five times each, from frozen aliquots. For these samples, the coefficient of

variation was 3.9% for a sample with 5.8 log₁₀ *C. difficile* DNA, 14.5% for a sample with 3.7 log₁₀ *C. difficile* DNA (5.7% excluding one outlier), and 12.3% for a sample with 2.0 log₁₀ *C. difficile* DNA.

DISCUSSION

Using a widely available PCR test, we prospectively quantitated *C. difficile* in clinical stool samples with toxin detected by immunoassay or cell cytotoxicity, and compared the results to samples without detectable toxins in which *C. difficile* was detected by PCR alone. With this approach, we show that toxin-negative samples routinely have 10¹–10⁴ less *C. difficile* than toxin-positive samples and most discrepancies between toxin tests and PCR are associated with a significant difference in *C. difficile* concentration (Figure 1A and 1B). This adds to the current understanding of the potential causes of toxin-negative, PCR-positive results but also implies that *C. difficile* quantitation could be used to predict toxin status clinically. To explore this, we plotted an ROC curve of the log₁₀ *C. difficile* DNA concentration versus the consensus toxin result from immunoassay and cytotoxin testing and found it to be a robust predictor of toxin status (Figure 2). For example, with an arbitrary cutoff of 4.10 log₁₀ *C. difficile*/mL feces selected for high sensitivity, 95% of toxin-positive samples and 70% of consensus toxin-negative samples were correctly classified by the real-time PCR *C. difficile* concentration alone (Figure 1B). In practice, the optimal quantitative cutoff may vary between *C. difficile* strains. We observed that samples with the hypervirulent BI/NAP1/027 *C. difficile* strain were 3 times more likely to be toxin-positive than samples with a non-027 strain and, when 027 samples were toxin-negative, they had a lower *C. difficile* concentration than toxin-negative samples with a non-027 strain. These observations support previously published data that BI/NAP1/027 *C. difficile* strains may produce toxins earlier or in greater amounts [9–11].

An important clinical question raised by this study and others is whether low-concentration, toxin-negative *C. difficile* cases represent CDI in patients that are missed by toxin tests (especially cytotoxin assays) or low level colonization in patients with another cause of diarrhea [3, 4, 12]. In our study, nearly a third of all samples (30.6%) had this test profile (i.e., toxin-negative, low-concentration *C. difficile* positive) indicating that the number of patients in the U.S. and internationally with such results is likely to be substantial. Toxin-negative patients rarely convert to toxin-positive (~1–3%) with repeat testing of subsequent stool samples but few studies have directly examined clinical characteristics and outcomes of toxin-negative patients [13–17]. In one study that did evaluate toxin-negative, *C. difficile*-positive patients clinically, 43/146 (29.5%) culture-positive patients were cytotoxin assay-negative similar to our study [18]. However, 40/43 (93.0%) of these patients had normal endoscopy and were clinically indistinguishable from controls except for a higher frequency of fever leading the authors to conclude that they were probably *C. difficile* carriers. Three patients (3/43; 7.0%) had pseudomembranes by endoscopy and CDI [18]. More recently, a large, prospective study of 12,420 stool samples that included some clinical data found that the presence of *C. difficile* toxins in fecal samples was significantly associated with a poor clinical outcome while culture positivity in the absence of detectable toxins was not associated with a clinical outcome worse than *C. difficile* negative samples [19, 20]. From this study, the investigators concluded that toxin-negative, *C. difficile*-positive patients were most likely carriers or “excretors” and United Kingdom National Health Service guidance documents for *C. difficile* testing have been updated to recommend two step algorithm using a sensitive nucleic acid amplification test (e.g., PCR or similar) or glutamate dehydrogenase (GDH) immunoassay for screening followed by a *C. difficile* toxin test to distinguish toxin-positive patients from toxin-negative patients [20]. Our data supports the conclusions of these two studies by showing that cytotoxin-negative patients have a lower concentration of *C. difficile* than toxin-positive patients and suggests that quantitation could be used to

predict toxin-status without independent toxin testing although this would need to be validated more extensively prior to clinical use. Important questions that would need to be addressed include the reproducibility of *C. difficile* quantitation given the heterogeneity of fecal samples, optimal quantitative cutoff(s) for different strains and correlation with clinical patient outcomes. We observed that quantitation was very reproducible (5%) at high *C. difficile* concentrations but more variable (15%) at middle and low concentrations but this needs to be evaluated with larger numbers of samples and diverse sample types.

In conclusion, our study builds on previous studies that have shown an association between the amount of *C. difficile* present in the sample and the likelihood that toxins will be detected directly [12, 21–23]. Uniquely, we used standard curves with a widely available real-time PCR diagnostic test to calculate the *C. difficile* fecal load in diarrheal stool samples and show that toxin-negative samples routinely have 10^1 – 10^4 less *C. difficile* than toxin-positive samples. Our results suggest that differences in the concentration of *C. difficile* between samples probably underlie most discrepancies between toxin tests and PCR implying that quantitation could be used clinically to predict toxin status. More studies are needed to determine the clinical significance of low concentration *C. difficile* in the absence of detectable toxin but it is possible that quantitation could be used to help distinguish patients with CDI from carriers with diarrhea due to other causes while still recognizing carriers for prevention of transmission. In short, we believe that *C. difficile* quantitation deserves further study as a potentially useful adjunct to current diagnostic tests.

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Fig. 1.

Fecal *C. difficile* concentrations of toxin-positive and toxin-negative stool samples by quantitative PCR. (A) Sample status after toxin A and B immunoassay (EIA) and real-time PCR. (B) Sample status after cell cytotoxicity testing of available EIA toxin-negative samples (N=55/61, 90.2% of toxin-negative samples tested). Circles (○) are toxin-negative samples. Squares (□) are toxin-positive samples. Open circles and squares are samples with non-027 *C. difficile* strains. Closed or solid circles and squares are samples with presumptive BI/NAP1/027 *C. difficile* strains. Red squares (open or solid) are samples that converted from toxin-negative to toxin-positive after cell cytotoxicity testing. Solid lines are medians. Dashed lines indicate the 4.1 log_{10} *C. difficile tcdB* DNA copies/mL cutoff discussed in the text.

Fig. 2. Receiver operating characteristic (ROC) curve for $\log_{10}$ *C. difficile tcdB* DNA copies per milliliter of feces versus fecal toxin status. Dashed lines indicate the 4.1 $\log_{10}$ *C. difficile tcdB* DNA copies/mL cutoff discussed in the text (95% sensitivity, 70% specificity).