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Ultrasensitive Detection of *Clostridium difficile* Toxins Reveals Suboptimal Accuracy by Toxin-Gene Cycle-Thresholds for Toxin Predictions

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ABSTRACT

The use of nucleic acid amplification tests (NAATs) for the diagnosis of *Clostridium* (*Clostridioides*) *difficile* infection (CDI) leads to overdiagnosis. To improve the clinical specificity of NAATs, there has been a recent interest in using toxin-gene cycle-thresholds (CT) to predict the presence and absence of toxins. Although there is an association between CT values and fecal-toxin concentrations, the predictive accuracy of the former is suboptimal for use in clinical practice. Ultrasensitive toxin immunoassays to quantify free toxins in stool offer a novel option for high-sensitivity fecal-toxin detection, rather than using surrogate markers for prediction.

COMMENTARY

Diagnosis and management of patients presenting with suspected *Clostridium* (*Clostridioides*) *difficile* infection (CDI) can be complex. Diagnosis is based upon clinical presentation combined with a choice of stool tests, including the detection of *C. difficile* toxins A (TcdA) and B (TcdB), which are the primary virulence factors causing clinical disease, and molecular (nucleic acid amplification) tests, such as polymerase chain reaction (PCR), which target a toxin gene. Recent advances have allowed for the quantification of TcdA and TcdB as well as assessment of toxin gene load in diarrheal fecal samples from patients with suspected CDI. When the concept of genomic load, determined by real-time PCR cycle threshold (CT), was first put forward, preliminary data demonstrated promise for using this tool to indirectly assess toxin load and hence to possibly predict disease severity and clinical outcomes (1–8). Recently, studies using quantitative ultrasensitive toxin assays are questioning the clinical utility of PCR beyond detection of toxin genes (9–11).

Diagnostic Tools with Different Targets

C. difficile infection (CDI) is a toxin-mediated disease and detection of free TcdA and/or TcdB in stool correlates with outcome and severity (12, 13), but currently available toxin enzyme immunoassays (EIAs) are hampered by poor sensitivity and lack of a quantitative readout. Also, assays measuring toxin in cell-culture based assays (cell cytotoxicity neutralization assay; CCNA) are subjective and have a long turnaround time (up to 48 hours). Detection of toxigenic organisms, either by nucleic acid amplification tests (NAATs, such as PCR) or toxigenic culture, is insufficient for differentiation between CDI cases and *C. difficile* carriers (who have symptoms not due to CDI) (12, 13). Notably, signs and symptoms in CDI cases and *C. difficile* carriers overlap considerably, especially in hospitalized (usually elderly) patients

with multiple co-morbidities and many possible causes of diarrhea (14). Furthermore, none of these test methods assess the quantity of toxin present.

Toxin EIAs were the mainstay of CDI diagnostics before NAATs for *C. difficile* toxin gene(s) became commercially available in 2009 (15). For clinicians, who may have experienced missing cases using toxin EIAs, NAATs offered a convenient rule-out of CDI. NAATs detect *C. difficile* organisms with the capacity to produce toxin and have high negative predictive values, but their low clinical specificity has significant effects on patient care and epidemiology.

Since the late 1990s, when CDI surveillance improved, incidence and severity of CDI cases have increased (16). This has been attributed to various causes, such as outbreaks of hypervirulent strains and increased transmission pressure, but also ascertainment bias (16). In parallel with the observed increasing disease rates, molecular methods for detection of toxin genes were introduced to clinical laboratories as a primary, first-line diagnostic tool. Reported CDI incidence increased rapidly, by up to 67% in certain regions, and >100% in individual healthcare centers, when testing methods changed from toxin EIAs to NAATs (17). The mentioned factors attributable to risk for CDI may facilitate an increased transmission, but cannot alone explain such dramatic changes in epidemiology. Reported disease incidence varies with type of laboratory method used for diagnosis (16), and to avoid overdiagnosis, CDI guidelines have recommended against using NAATs as standalone tests in unselected patient populations (18, 19).

PCR CT Values for Prediction of Toxin

To expand the clinical utility of NAATs beyond the limitations associated with toxin-gene detection, there has been a recent interest in determining whether real-time PCR CT values can predict the

66 presence or absence of *C. difficile* toxin. In a Dutch multicenter study, it was shown that patients with
67 NAAT-positive and toxin EIA-positive samples had lower *tcdB* CT values (hence higher genome load) than
68 subjects with NAAT-positive and toxin-negative stool. When using optimal CT cutoff values (25.3 and
69 27.0 in each of two study sub-cohorts) to estimate the accuracy of CT values for prediction of toxin EIA
70 status, the area under the receiver operating characteristic (AuROC) curves were 0.826 and 0.854,
71 respectively. Prediction of toxin EIA results was accurate for 78.9% and 80.5% of the samples in each
72 sub-cohort. The authors concluded that CT values could serve as predictors of toxin status, but noted
73 that additional toxin testing was still needed due to poor accuracy (1).

74 In another study, *tcdB* CT values were analyzed in PCR-positive samples reflexed to toxin EIA and CCNA.
75 Using EIA as the reference method, a *tcdB* CT cutoff of 26.4 detected toxin-positive samples with a
76 sensitivity, specificity, positive predictive value, and negative predictive value of 96.0%, 65.9%, 57.4%,
77 and 97.1%, respectively. Using both EIA and CCNA as the reference method (toxin present if either EIA
78 or CCNA is positive) , the specificity was improved to 78.0%. It was concluded that PCR may be used to
79 predict toxin-negative stool samples (2). Further analysis at the same institution showed that PCR-
80 positive patients with CT values above the cutoff had similar outcomes regardless of treatment status
81 (54 treated and 43 untreated), and that reporting of predicted toxin status based on CT value reduced
82 treatment of PCR-positive patients by 15%, with no increase in adverse outcomes (20).

83 When performing toxin-EIA testing in 1,650 PCR-positive patient samples, a *tcdB* Ct value of $\leq$26 was
84 associated with EIA positivity, higher mortality, and CDI severity (8). Seventy-two percent of patients
85 with CT values 18-21 had severe/recurrent CDI, and 59% of mild cases with CT values 18-21 had
86 treatment failure with first-line therapy. By contrast, 92% of the patients with CT values 35-37 had mild
87 CDI and responded to treatment. However, *tcdB* Ct values of ≤26 missed 28% of toxin-EIA positive

patients and the authors suggested that a CT of ≤26 could be used as an adjunct in CDI testing algorithms and to guide reporting.

Other studies have demonstrated differences in *tcdB* CT values between groups positive and negative by toxin EIA, and estimated toxin-EIA positivity with 79.3% sensitivity and 83.6% specificity (AuROC 0.848) when using a cutoff of 26.3 (3). Using CCNA as reference method, *tcdB* CT values could predict 77% of CCNA-positive cases in patients with cancer when using a CT cutoff of 28.0, and 91% and 100% of severe and complicated CDI episodes, respectively (4). In addition, CT values in patients with CDI were significantly lower than in excretors, i.e. patients with diarrhea who have toxigenic *C. difficile* in stool but with no detectable free toxin (5). An inverse correlation between CT and *C. difficile* fecal loads (Spearman -0.70), as estimated by using quantitative culture, has also been reported (6), as well as an association between the amount of *C. difficile* present in the sample and the likelihood that toxins will be detected directly (AuROC 0.921 for *tcdB* DNA copy number versus toxin result) (7), suggesting that CT could be used as a surrogate marker for bacterial load and disease activity.

Clinical Use of CT Values Concerning

Scientists at King's College London also observed a significant correlation between *tcdB* CT values and toxin-EIA positivity, but drew a more cautious conclusion regarding implementation in clinical practice (21). In their study on over 1400 patients, CT values were lower in samples positive by toxin EIA compared with toxin-negative samples, suggesting a higher organism load. The AuROC curve, 0.806, was similar to the one generated by Kamboj *et al* (4), and the sensitivity and specificity were 83.1% and 67%, respectively, at an optimal CT value threshold of 27.0. However, the authors observed a significant overlap of CT values in those that were positive and negative by toxin EIA, and concluded that this made it difficult in practice to use *tcdB* CT values to definitively categorize individual patients in this way (21).

In a study on 1,281 PCR-positive samples, a *tcdB* CT ≤25 was significantly associated with a toxin-positive result, as assessed using CCNA, with 51.3% sensitivity, 87.5% specificity, and 83.9% positive predictive value for presence of toxin (AuROC 0.831). CT values were lower in toxin-positive samples than in toxin-negative samples (median 24.9 vs 31.6) but did not differ between patients with or without a CDI recurrence. There was an association between both *tcdB* CT value and mortality and various signs of disease severity, and values were lower in patients who died than in survivors. The conclusions from the study were that due to the relatively low sensitivity and specificity for confirmation of detection of toxin, *tcdB* CT values cannot be used as a standalone test (22).

Studies estimating accuracy of CT values for toxin prediction use either toxin EIA or CCNA as references standards. Both tests have limitations, including poor analytical sensitivity and a non-quantitative format for toxin EIAs and a detection limited to primarily TcdB by CCNA. In addition, both tests have binary interpretations. With the advent of quantitative ultrasensitive toxin immunoassays, capable of quantification at very low concentrations, from picogram-per-millimeter levels (11, 15), an accurate assessment of toxin load can now be determined and the clinical value of using *tcdB* CT values to indirectly predict toxin can be further evaluated (9, 11, 15). In a recent study using PCR and an ultrasensitive toxin assay, multiple patients with CT values >26.4 had detectable stool toxin, including above analytical thresholds for EIA (~1000 pg/mL) and CCNA (TcdB of ~100 pg/mL) (10).

In a recent study using ultrasensitive Single Molecule Counting technology for toxin quantification (Singulex Clarity C. diff toxins A/B assay), there was also a significant inverse correlation between *tcdB* CT values and toxin concentrations (Spearman -0.64) in 211 patients with suspected CDI. However, 16 toxin-negative samples (<12.0 pg/mL) had *tcdB* CT values <27.0 (25.0% of all PCR+/toxin- samples), and 21 toxin-positive samples had CT values >27.0 (14.3% of all PCR+/toxin+ samples) (11). Similarly, in a recent study on 207 patients with PCR-positive samples, there were 18 samples toxin-negative by Clarity

with *tcdB* CT values <27.0 (22.8% of all PCR+/toxin- samples), and 36 toxin-positive samples with CT values >27.0 (14.3% of all PCR+/toxin+ samples) (9).

Possible Ways Forward

Guided by studies showing clinical utility, some laboratories may now consider implementing *C. difficile* toxin gene(s) CT values in CDI diagnostics, for prediction of free toxin and estimation of disease severity for treatment guidance. However, until the recent introduction of ultrasensitive toxin assays, no technology has been available for toxin measurements at picogram-per-milliliter levels. Presence and absence of *C. difficile* toxins have been defined by EIA or CCNA positivity. Thus, ultrasensitive immunoassays can be used to further evaluate the potential of *tcdB* CT values to predict the presence of fecal toxin. CT values, at the proposed cutoffs, do not detect all samples with high toxin concentration, not even those with very high concentration (above the EIA and CCNA cutoffs) (10). Although there is a correlation between *tcdB* CT values and toxin concentration, the accuracy is suboptimal for use in clinical practice. There is a significant risk of misclassifying patients and either treating incorrectly or inappropriately refraining from treatment. As reported in multiple studies using ultrasensitive toxin assays, a large proportion of patients with high toxin concentrations would have been misinterpreted as having undetectable toxin, if *tcdB* CT values had been used clinically. For many clinicians, such a high miss rate would be unacceptable.

It is important to note the contribution of host factors in a discussion about CDI diagnosis. We note that CDI and the influence of host factors have been established previously. Kyne *et al* showed that asymptomatic *C. difficile* carriers had high serum levels of toxin-A IgG, but that patients who became colonized by *C. difficile* but who had low levels of toxin-A IgG in serum had a much greater risk of CDI (23). The same group later showed that a serum antibody response to toxin A, during an initial episode

of CDI, was associated with protection against recurrence (24). Further studies are needed to understand the clinical significance of both low and high toxin concentrations, as detected by ultrasensitive assays. If toxins in low concentrations are deemed clinically meaningful, *tcdB* CT value cutoffs based on low-sensitive toxin assays will not be useful. *tcdB* CT values as surrogate markers for *C. difficile* toxin status provide unacceptable accuracy in terms of predicting toxin-positive patients in studies using conventional EIAs or CCNA; such observations are reinforced by studies using ultrasensitive toxin detection. Measurements of free toxins in stool can now be achieved at levels fulfilling the need for both sensitivity and specificity. With the development of automated, ultrasensitive toxin assays, the use of standalone NAATs and multistep algorithms in CDI diagnostics could potentially be replaced with a single, direct test for free toxin.

Conflict of Interest

Johanna Sandlund is an employee of Singulex, Inc.

Mark Wilcox has provided consultancy advice to multiple CDI diagnostic companies, including Singulex, Inc.

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