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COMMENTARY

Ultrasensitive Detection of *Clostridium difficile* Toxins Reveals Suboptimal Accuracy of 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 (C_Ts) to predict the presence and absence of toxins. Although there is an association between C_T 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.

KEYWORDS C_T value, *Clostridium difficile*, toxin

The 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 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 (C_T), 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 the 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); however, currently available toxin enzyme immunoassays (EIAs) are hampered by poor sensitivity and the 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 h). The detection of toxigenic organisms, either by nucleic acid amplification tests (NAATs; such as PCR) or toxigenic culture, is insufficient for differentiating between CDI cases and *C. difficile* carriers (who have symptoms not due to CDI) (12, 13). Notably, the signs and symptoms in CDI cases and *C. difficile* carriers overlap considerably, especially in hospitalized (usually elderly) patients with multiple comorbidities 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 the *C. difficile* toxin gene(s) became commercially available in 2009 (15). For clinicians, who may have

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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, the 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 the detection of toxin genes were introduced to clinical laboratories as a primary first-line diagnostic tool. The reported CDI incidence increased rapidly, by up to 67% in certain regions and >100% in individual health care 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. The reported disease incidence varies with the 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 C_T 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 C_T values can predict the presence or absence of *C. difficile* toxin. In a Dutch multicenter study, it was shown that patients with NAAT-positive and toxin EIA-positive samples had lower *tcdB* C_T values (hence, higher genome load) than subjects with NAAT-positive and toxin-negative stool. When using optimal C_T cutoff values (25.3 and 27.0 in each of two study subcohorts) to estimate the accuracy of C_T values for the prediction of toxin EIA status, the area under the receiver operating characteristic (AUROC) curves were 0.826 and 0.854, respectively. The prediction of toxin EIA results was accurate for 78.9% and 80.5% of the samples in each subcohort. The authors concluded that C_T values could serve as predictors of toxin status but noted that additional toxin testing was still needed due to poor accuracy (1).

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

When performing toxin EIA testing in 1,650 PCR-positive patient samples, a *tcdB* C_T value of ≤26 was associated with EIA positivity, higher mortality, and CDI severity (8). Seventy-two percent of patients with C_T values of 18 to 21 had severe/recurrent CDI, and 59% of mild cases with C_T values of 18 to 21 had treatment failure with first-line therapy. In contrast, 92% of the patients with C_T values of 35 to 37 had mild CDI and responded to treatment. However, *tcdB* Ct values of ≤26 missed 28% of toxin EIA-positive patients, and the authors suggested that a C_T of ≤26 could be used as an adjunct in CDI testing algorithms and to guide reporting.

Other studies have demonstrated differences in *tcdB* C_T 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* C_T values predicted 77% of CCNA-positive cases in patients with cancer when using a C_T cutoff of 28.0 and 91% and 100% of severe and complicated CDI episodes, respectively (4). In addition, C_T 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 C_T 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 of 0.921 for *tcdB* DNA copy number versus toxin result) (7), suggesting that C_T could be used as a surrogate marker for bacterial load and disease activity.

CLINICAL USE OF C_T VALUES IS CONCERNING

Scientists at King's College London also observed a significant correlation between *tcdB* C_T values and toxin EIA positivity but drew a more cautious conclusion regarding implementation in clinical practice (21). In their study on over 1,400 patients, C_T values were lower in samples positive by toxin EIA than in 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 C_T value threshold of 27.0. However, the authors observed a significant overlap of C_T values in those that were positive and negative by toxin EIA and concluded that this made it difficult in practice to use *tcdB* C_T values to definitively categorize individual patients in this way (21).

In a study on 1,281 PCR-positive samples, a *tcdB* C_T of ≤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). C_T values were lower in toxin-positive samples than in toxin-negative samples (median, 24.9 versus 31.6) but did not differ between patients with or without a CDI recurrence. There were associations between both *tcdB* C_T 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 the confirmation of detection of toxin, *tcdB* C_T values cannot be used as a standalone test (22).

Studies estimating the accuracy of C_T values for toxin prediction use either toxin EIA or CCNA as references standards. Both tests have limitations, including poor analytical sensitivity and a nonquantitative 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, which are 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* C_T 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 C_T values of >26.4 had detectable stool toxin, including values higher than analytical thresholds for EIA (~1,000 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* C_T values and toxin concentrations (Spearman, −0.64) in 211 patients with suspected CDI. However, 16 toxin-negative samples (<12.0 pg/ml) had *tcdB* C_T values of <27.0 (25.0% of all $PCR^+/toxin^-$ samples), and 21 toxin-positive samples had C_T values of >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* C_T values of <27.0 (22.8% of all $PCR^+/toxin^-$ samples) and 36 toxin-positive samples with C_T values of >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) C_T values in CDI diagnostics for the 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. The 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* C_T values to predict the presence of fecal toxin. C_T values, at the proposed cutoffs, do not detect all samples with high toxin concentrations, not even those with very high concentrations (greater than the EIA and CCNA cutoffs) (10). Although there is a correlation between *tcdB* C_T 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* C_T 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* C_T value cutoffs based on low-sensitive toxin assays will not be useful. *tcdB* C_T 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.

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