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Potential of Real-time PCR threshold cycle (C_T) to predict presence of free toxin and clinically relevant *C. difficile* infection (CDI) in patients with cancer

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Abstract

Objectives—*Clostridium difficile* infection (CDI) is a toxin-mediated disease. Oncology patients are at increased risk for developing CDI. Diagnosis of CDI by PCR has led to misclassification of some *C. difficile* carriers as CDI cases. We determined if an optimized *C. difficile* PCR cycle threshold value (C_T) could reliably predict presence of free toxin, and in turn improve the utility of PCR in detecting clinically relevant CDI in oncology patients.

Methods—183 consecutive patients positive for *C. difficile* by the Xpert *C. difficile* were additionally tested using the cell culture cytotoxicity neutralization assay (CYT) and enzyme immunoassays (EIA). C_T values at diagnosis and relevant clinical information were recorded. Receiver operating characteristic (ROC) curve was used to assess predictive validity and to find optimal C_T for CYT positive cases. Severity of CDI was assessed by blinded charts review.

Results—Using CYT as the reference, ROC-derived Youden cut-off C_T of 28.0 predicted 77% cytotoxin positive cases, and 91% and 100% of severe and complicated CDI episodes respectively. The median C_T values for non-severe, severe, and complicated CDI episodes were 28.0, 24.5 and 22.5 respectively ($p=0.005$).

Conclusions—Lower C_T value of the Xpert *C. difficile* PCR was associated with the presence of toxin and increased CDI severity. C_T values may be beneficial in interpreting positive *C. difficile* PCR results.

Keywords

Clostridium difficile infection; Real-time PCR; Oncology patients; Cytotoxin neutralization assay

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Clostridium difficile is the most common infectious cause of healthcare-associated diarrhea. In acute care settings, colonization with toxigenic *C. difficile* (TCD) can occur in up to 15 % of patients and the risk of *C. difficile* infection (CDI) is estimated to be between 1–2 % (1, 2). Clinical syndromes associated with CDI range from mild diarrhea, to severe disease including toxic megacolon. As one of the leading causes of healthcare-associated infections (HAI), CDI is associated with high morbidity, and contributes substantially to healthcare associated costs (3, 4). In the United States, high rates of CDI prompted a national effort to reduce the burden of *C. difficile* associated disease. Public reporting of CDI rates is now mandated by many states and linked to payment reduction penalties as part of the Hospital-Acquired Condition Reduction Program (5).

The first PCR for the diagnosis of CDI was approved by the US Food and Drug Administration (US FDA) in 2008 and widely adopted due to its high analytic sensitivity and low complexity, despite a lack of accompanying clinical experience. In the years to follow, the potential misdiagnosis of TCD carriers as CDI cases was recognized as a major consequence of PCR with wide ranging implications especially due to expanded HAI reporting (6, 7). There is now growing evidence to support the notion that PCR positive cases of CDI without detectable toxin may not require treatment, regardless of the presence of diarrhea, particularly if not severe (6, 7). It is also increasingly apparent that carriers of TCD contribute towards hospital-based transmission and are an important target for control efforts, although their precise role and the optimal steps that would prevent onward spread of disease from TCD carriers remains unclear at this time (6–10).

To address the limitations of PCR, and its associated increased costs, multi -step testing algorithms were recently adopted by the European guidelines as the new standard of *C. difficile* testing (11). Although such a strategy does not overcome the limitations of single step PCR, many US centers have implemented the dual testing algorithm starting with antigens testing to counter the increase in reportable HAI rates observed with PCR use. Data to support the utility and clinical rationale for a two step testing strategy are incomplete (7, 12). For laboratories that use glutamate dehydrogenase (GDH) and/or Toxin A/B enzyme immunoassays (EIA) as a first step followed by PCR, specimens that are GDH positive/toxin negative will still detect TCD carriers as CDI (specificity issue). Similarly, PCR followed by confirmatory toxin EIA will miss clinically relevant CDI cases (sensitivity issue) if only EIA results are reported.

Another unique approach to tackle the challenge of CDI diagnosis is to leverage the quantitative capability of PCRs (13, 14). For real-time PCR tests, cycle threshold (C_T) values inversely correlate with the log amount of target in the sample. Using C_T as a quantitative metric, our group has previously demonstrated that higher bacterial loads (low C_T) in the stool of patients infected with *C. difficile* correlate strongly with detection of free toxin by CYT (15, 16). Other groups have similarly evaluated the utility of C_t values (primarily Xpert *C. difficile* PCR) to predict the presence of free toxins or its association with CDI outcomes (17, 18). However, no studies have been performed in a primarily oncology patient population, a group at high risk for CDI. If the clinical utility of this approach is validated, routine reporting of C_T values, along with an interpretive comment,

could prove to be beneficial to clinicians and infection control personnel in interpreting positive *C. difficile* PCR results.

In this current study, we hypothesized that CT value cut-offs can be established for PCR assays to reliably predict CYT positive cases, which may be more relevant from a clinical and epidemiologic standpoint.

Methods

Hospital Setting and Study Design

This study was performed at Memorial Sloan Kettering Cancer Center (MSKCC), a 473-bed tertiary care cancer hospital. All stool samples that tested positive by the *C. difficile* Xpert PCR/Epi PCR between July 1, and September 22, 2016 were prospectively collected. Negative stool samples on the Xpert PCR assay do not have a C_T value reported by the instrument. The following tasks were performed for PCR positive specimens: 1) Retrieval of readily available C_T values from the initial diagnostic run; 2) Simultaneous testing of all PCR positive stool specimens with CYT, GDH, and toxin A/B EIA; and 3) Comparison of underlying risk factors and outcomes for all PCR positive cases based on the combined performance of all diagnostic assays and C_T values.

Laboratory Tests

All unformed stool samples were tested upon receipt in the laboratory per routine protocol. The C_T value for each case was recorded retrospectively by accessing the instrument archives. Following completion of routine testing, stool samples were frozen at -20°C until additional testing could be performed (mean [range]: 7 days [1–19] at 4°C). All samples were tested by the following three methods: GDH antigen EIA (GDH EIA; C. DIFF CHEK-60, Techlab, Blacksburg, VA), *C. difficile* toxin A and B (Tox A/B) antigen EIA (Tox A/B EIA; *C. difficile* Tox A/B II, Alere, Orlando, FL) and CYT (*C. difficile* toxin/antitoxin kit; Techlab, Blacksburg, VA). CYT and GDH tests were performed as previously described (19). The Tox A/B EIA was performed according to manufacturer's instructions. The technologist performing the CYT and EIA testing was blinded to PCR C_T values for the study samples. Multi-locus sequence typing (MLST) of all *C. difficile* strains recovered in culture was performed as previously described (20).

Clinical Data Collection

The following clinical data were collected: age, gender, underlying disease, transplant, chemotherapy, major surgery 30 days prior to onset, laxative use within previous 48 hours (inpatients only), length and duration of antibiotic therapy (LOT and DOT) within 30 days preceding the CDI event, including outpatient antibiotic use. Nosocomial or community based acquisition of infection and past CDI events, among study participants were obtained from the Infection Control database (CKM, Canada). CDI cases were categorized as non-severe, severe, and complicated disease. Complicated CDI was defined by the presence of toxic megacolon or ileus or need for colectomy. Histopathologic corroboration was done to confirm colectomy as a complication of CDI. Disease severity determination was based on guidelines from SHEA and IDSA (21) and any of the following: white blood cells (WBC) >

15 K/ μ L, acute kidney injury (> 50 % increase in serum creatinine from baseline, ICU admission attributed to CDI symptoms, severe abdominal symptoms with evidence of colitis on Computed tomographic (CT) scan. Cases were reviewed independently by two board certified Infectious Disease Physicians (EVR and MK) who were blinded to the results of other diagnostic assays.

Statistical analysis

Summary statistics were calculated as percentages and means or medians and differences were evaluated via Chi-squared tests and t tests, except in cases of sparse cells and non-normality, when Fisher's exact and Kruskal-Wallis tests were used. To calculate summary statistics by CYT positivity and disease severity, only the first sample was used. Sample size calculations to determine an optimal C_T value cut off were made with the assumption of a 0.95 sensitivity and 0.90 specificity of the CYT using methods proposed by Jensen et al (22). Receiver operating characteristic (ROC) curve was used to assess predictive validity and to find optimal C_T cut off values. The optimal C_T value threshold for determining *C. difficile* positivity by CYT was calculated using Youden's index ($J = \text{sensitivity} + \text{specificity} - 1$) (23). Differences with a p-value of <0.05 were considered significant. All analyses were conducted in SAS 9.4, GraphPad Prism 7, or R 3.1.2.

Ethical Considerations

The study was approved by the MSKCC Institutional Review Board.

RESULTS

Laboratory tests

Sample size calculation estimated that between 174 and 200 positive samples were needed to determine optimal C_T value. 1,638 specimens were tested by PCR during the study period of which 202 stools from 183 unique patients were positive by PCR. The detection rates for Tox A/B, CYT and GDH were 35.5% (65/183), 59.6% (109/183), and 92.9 % (170/183), respectively. The median C_T value for all positive study samples was 28.0 [range 19.7–36.8]; samples that were positive by PCR and negative by CYT had a median C_T value of 31.8 [range 21.1–36.8], compared to a median C_T value of 25.3 [range: 19.7–36.1] among samples positive by PCR and CYT ($p < 0.001$) (Figure 1). EIA data presented reflect samples that were both GDH positive and Tox A/B positive samples (median C_T value of 24.2 [range: 19.7–34.9]).

An ROC curve was generated using CYT as the reference method. The diagnostic accuracy as shown by the area under the curve (AUC) was good at 0.83 (Figure 2). The AUC and maximum Youden index value (0.51) yielded an optimal C_T value threshold of 28.0, with a corresponding sensitivity and specificity of 77% (95% CI, 68–83%) and 74% (95% CI, 64–82%) and positive and negative predictive values of 81% (72–87%) and 70% (60–78%) respectively (Table 1). When using EIA as the reference method, a C_T value threshold of 28.0 had a sensitivity of 91% (95% CI, 81–97%) and specificity of 68% (95% CI, 59–76%) (Data not shown).

Baseline Characteristics

Upon examining the study cohort by CYT positivity, no significant differences were detected in the demographic characteristics, underlying cancer, history of stem cell transplant, laxative use within 48 hours and chemotherapy or major surgery in preceding 30 days (Table 1). CYT positive cases had greater antibiotic exposure. Days of therapy (DOT) and length of therapy (LOT) in 30 days preceding the CDI event among CYT + and CYT- cases were: DOT = 1 [IQR =0–5.75] vs 5 [IQR 0–13] $p=0.004$; LOT=1 [IQR 0–4.75] vs 4 [IQR 0–9]; $p=0.002$. The median duration of hospitalization at the time of CDI diagnosis was longer for CYT positive cases but not statistically significantly different. Nosocomial cases were more likely to be CYT positive when compared to community-acquired cases.

For genotyping, toxigenic *C. difficile* was successfully retrieved in culture from 145/183 samples (79%), these included 97/109 CYT positive (recovery rate 89%), and 48/64 CYT negative samples (recovery rate 65%) (Data not shown). The median C_T for culture positive cases was significantly lower than culture negative cases (26.6 and 32.2 respectively; $p<0.001$). CYT positivity was significantly higher in infections due to hyper virulent or emerging *C. difficile* strains; 80 % for ST1, 100 % for ST11 and 80 % for ST8, when compared with endemic (ST2 or 42; 68% and 41 % were CYT positive respectively) or sporadic strains (48 %) [$p=0.02$]. Past history of CDI did not influence CYT positivity up to 12 months from the previous CDI episode.

C_T value and CDI disease severity by diagnostic test

168 (91.8%) patients were categorized as non-severe (Table 2). Eleven patients were severe (6%); and four (2.2%) were complicated. The median C_T values for non-severe, severe, and complicated CDI episodes were 28.0, 24.5 and 22.5 respectively ($p=0.005$). The four patients with complicated CDI included two patients with toxic megacolon, one among these cases required colectomy. The other two patients developed ileus as a complication from CDI. All four cases were detected by CYT and EIA with C_T values ranging from 22.4–25.3. Among the eleven severe cases, six were EIA negative, and two were CYT negative. For the two CYT negative cases, C_T values were 25.5 and 27.1. The C_T value for severe cases ranged from 19.7–33.5. Among the severe episodes was a case of a 46 year old neutropenic patient with Ewing Sarcoma who was transferred from another facility with significant clinical symptoms (> 10 bowel movements per 24 hours), and radiographic evidence of diffuse colitis on scan. He was retested upon transfer to our center. A C_T value of 33.5 was only available for PCR testing done following 48 hours of CDI therapy. Excluding this case, the C_T value among those with severe episode of CDI ranged from 19.7–28.6.

19 [10.4%] patients developed recurrence within 8 weeks of the study index episode. There was no relationship between C_T value and CYT positivity among patients who relapsed within this time frame compared to those that did not (data not shown).

DISCUSSION

We have demonstrated a statistically significant association between C_T values at the time of CDI diagnosis and detection of free toxin as demonstrated by CYT; we also have shown a

statistically significant association of severe and complicated CDI with lower C_T values when compared with non-severe episodes. As noted, all four cases with complicated CDI were CYT and EIA positive with significantly lower C_T compared to severe and non-severe episodes.

A Youden cut-off value C_T of 28.0 accurately detected all but one case (C_t value of 28.6) of severe CDI episodes, including two cases missed by CYT (C_T 25.2 and 27.1 respectively) as confirmed by medical review of these cases. The performance of toxin EIA in our study was suboptimal—missing half of CYT positive non-severe episodes and 30 % of all severe episodes. Collectively these findings demonstrate the promise of the Xpert *C. difficile* PCR C_T value in optimizing the positive predictive value of PCR for clinically relevant CDI, especially for laboratories using PCR as the sole method for CDI diagnosis.

Detection of toxin negative cases is variable between molecular assays depending on the analytic sensitivity; however it has been shown by more than one assay to correlate with stool bacterial burden. Using the Gen Xpert *C. difficile* assay, Leslie et al. compared PCR with toxigenic culture, toxin EIA, and CYT; their findings revealed that samples that were toxin positive had a 1 to 4 $\log_{10}$ 4 higher DNA load when compared with toxin negative samples (24). ROC for quantitative bacterial load (copies/ml) versus EIA+CYT+ demonstrated an AUC of 0.92 (95% CI 0.87–0.97). Quantitative assessment was extrapolated from standard curves run with each lot of testing. A similar report from Dione et al used the BD Gene Ohm to further prove an inverse linear relationship between higher stool bacterial load and C_T (13). In our study, the AUC was 0.83, using C_T value only with CYT+ as the reference assay.

The clinical implication of bacterial load at the time of diagnosis has not been examined thoroughly; only a limited number of reports have scrutinized the performance of various diagnostic assays by means of quantitative thresholds, and its relationship with clinical severity of CDI (6, 15, 17, 18) and none were performed exclusively in patients at high-risk for developing CDI. Polage et al established that among patients that test positive by PCR, there is a statistically significant difference in $\log_{10}$ *C. difficile* DNA copies/ml when samples were examined by toxin EIA results (median load for positive vs negative; 7.3 and 4.9 respectively; $p < 0.001$) (6). Our group has previously demonstrated that recurrent CDI is more common in patients that test positive by CYT and PCR when compared to cases positive by PCR only. This study was conducted at a time of peak NAP1 (ST1) prevalence at our center; the findings had also simultaneously revealed a higher CYT positivity rate among NAP1 infections, and established an association between CYT positivity and C_T values using two different PCR methods (15). Using an Xpert *C. difficile* PCR, Reigadas et al calculated a C_t value threshold of 23.5 to correlate with a high-risk for poor outcome CDI (18). Our current study, performed at a single cancer center, identified a median C_t value of 22.5 for patients with complicated CDI, suggesting that the data from our study may be similar to data obtained in a general patient population. Furthermore, Senchyna et al showed that patients' immune status did not impact the median C_t value (17). In their study, Senchyna et al identified that a threshold value of 26.35 had a sensitivity and specificity of 96% and 66% respectively for predicting the presence of free toxin when using a rapid membrane EIA test as the reference method. In our study, when using a toxin A/B plates

EIA as the reference method, a threshold value of 28.0 had a sensitivity and specificity of 91% and 68% respectively for predicting the presence of free toxin (data not shown). Differences in testing algorithms, patient population and test methodologies may explain the observed differences in threshold values between the two studies.

Compared to these previous reports, the strengths of our current study are that we inferred bacterial burden directly from the threshold cycle, suggesting that C_T is a readily available, simple and valuable adjunctive diagnostic tool that can be used to improve the specificity of PCR based *C. difficile* assays without extra burden to the existing laboratory workflow. Furthermore, although PCR tests reagents are often more costly than toxin assays, published studies have shown potential cost benefits of PCR testing alone compared to two-steps algorithms (19, 25). Most importantly, we confirm a statistically significant relationship between clinical severity and CYT positivity, and identify a C_T value of less than 28.0 as a predictor of CYT positive CDI.

An unexpected and unexplained finding from our study was a strong association between toxin positivity and previous antibiotic exposure suggesting a potential dose response between overall antibiotic exposure and severity of CDI. This possibility however will require substantial additional exploration to understand.

This was a proof of principle study. Some of the limitations of our study are listed below. First, the overall number of patients with severe and complicated diseases was low and larger study cohort that includes all patients tested for *C. difficile*, and not just PCR positive patients, will be required to validate the established threshold. Data presented in this study are based on an off-label use of the Xpert *C. difficile* PCR, which is FDA approved as a qualitative assay only. Therefore, further validation will be necessary to validate the use of C_t value as an adjunct to clinical decision making. Testing of samples and analysis was conducted at a single tertiary care cancer center and the findings may not be generalizable to other healthcare settings. The intra-assay reproducibility of the Xpert *C. difficile* C_t value was not addressed in this study although it has been demonstrated by others (17). Studies with larger, diverse populations are required to conclusively prove the association between C_T and disease severity. Although the proposed C_T cut off reliably detected hyper-virulent and emerging strains, our study was not specifically designed to address the role of C_T in estimating contagiousness. Finally, the inability to obtain a quantitative assessment from some of the other FDA approved PCRs somewhat limits the broad application of this approach and data obtained with the Xpert PCR might not hold true for other PCRs. However, quantitative parameters are a fundamental component of molecular tests and development of assay specific standards is a viable option.

Our study findings provide additional evidence that lower C_T cut offs for the Xpert *C. difficile*/Epi PCR could be established to improve the likelihood of detecting clinically relevant CDI. Routine reporting of C_T should be considered by laboratories using stand alone Xpert *C. difficile* PCR testing for CDI diagnosis as an adjunctive tool for clinician and infection control personnel to interpret positive PCR results.

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Highlights

- CDI is a toxin mediated disease
- qPCR cycle threshold cycle (C_T) may predict the presence of toxin and CDI severity
- A C_T of 28.0 (using cell culture toxin assay as reference) predicted 77% cytotoxin positive cases
- A C_T of 28.0 predicted 91% and 100% of severe and complicated CDI cases respectively

Figure 1. Median Cycle Threshold (C_T) value by diagnostic tests (n=183)

PCR only: Samples positive only by Polymerase Chain Reaction (n=74), Cytotoxin: Samples positive by Cell Culture Cytotoxin neutralization test (n=109), EIA: Sample positive by Enzyme-linked Immunoassay (n=65). The horizontal line represents the median C_T value for each diagnostic test.

Figure 2. Receiver operating characteristics curve (ROC) to assess performance of threshold cycle (C_T) from PCR run at the time of diagnosis (n=202). Detection of free toxin by Cell Culture Cytotoxin neutralization (CYT) test serves as reference method.

Table 1

Performance characteristic of various cycle threshold values using CYT as reference method

C _T value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
21	7 (4–13)	99 (94–100)	100 (60–100)	44 (37–51)
23	24 (17–33)	99 (94–100)	96 (83–100)	49 (41–56)
25	51 (42–60)	93 (85–97)	91 (81–96)	58 (50–66)
28*	77 (68–83)	74 (64–82)	81 (72–87)	70 (60–78)
31	87 (80–92)	51 (40–61)	71 (63–77)	74 (62–84)
37	100 (97–100)	0 (0–4)	58 (51–64)	N/A

CI: Confidence Interval

Table 2Clinical Characteristics by Cell Cytotoxicity Assay Results ^a

	Cytotoxin negative	Cytotoxin positive	P value
Number of Patients	74	109	
Age [median (IQR)]	59 [45, 70]	61 [37, 73]	0.83
Pediatric case (%)	8 (10.8)	12 (11.0)	1
Sex [male (%)]	36 (48.6)	60 (55.0)	0.48
Type of tumor (%)			0.21
Solid tumor	35 (47.3)	63 (57.8)	
Liquid tumor	39 (52.7)	46 (42.2)	
HSCT (%)	13 (17.6)	20 (18.3)	1
Chemotherapy (within previous 30 days)	42 (56.8)	54 (49.5)	0.42
Surgery (within previous 30 days)	23 (31.1)	48 (44.0)	0.11
Laxative use within previous 48 hours for inpatients only (%)	7 (9.5)	14 (12.8)	0.639
Antibiotic- Length of therapy^b (median [IQR])	1.00 [0.00, 4.75]	4.00 [0.00, 9.00]	0.002
Antibiotic- Duration of therapy^b (median [IQR])	1.00 [0.00, 5.75]	5.00 [0.00, 13.00]	0.004
Infection Origin (%)			0.09
Community Acquired Inpatient	19 (25.7)	23 (21.1)	
Community Acquired Outpatient	35 (47.3)	38 (34.9)	
Nosocomial	11 (14.9)	33 (30.3)	
Recurrent	9 (12.2)	15 (13.8)	
Days to <i>C. difficile</i> if inpatient (median [IQR])	4.00 [2.00, 10.00]	6.00 [3.00, 10.00]	0.21
MLST profile (ST type) (%)			0.02
1	1 (1.4)	4 (3.7)	
11	0 (0.0)	7 (6.4)	
2	6 (8.1)	13 (11.9)	
42	7 (9.5)	5 (4.6)	
8	3 (4.1)	13 (11.9)	
Other ST types	57 (77.0)	67 (61.5)	
Nontypable (lack of growth)	16 (22)	12 (11)	
History of CDI within 12 months (%)	14 (18.9)	21 (19.3)	1
Recurrent CDI within 8 weeks of current sample (%)	9 (12.2)	10 (9.2)	0.69
EIA positive (%)	7 (9.5)	58 (53.2)	<0.001
C_T Value [median (range)]	31.8 (21.1–36.8)	25.3 (19.7–36.1)	<0.001

^aTo calculate summary statistics, only the first sample was used.^bwithin 30 days preceding the CDI

Table 3

CDI severity and Cycle Threshold Value median by Diagnostic tests *

Clinical Severity	Non severe	Severe	Complicated
	N= 168	N=11	N=4
			p=0.014
PCR+/CYT-/EIA-	65	2	0
PCR+/CYT-/EIA+	7	0	0
PCR+/CYT+/EIA-	48	3	0
PCR+/CYT+/EIA+	48	6	4
CT value median (range)	28.0 (20.0–36.8)	24.5 (19.7–28.6) **	22.5 (22.4–25.3)
			p=0.005

PCR: Polymerase Chain Reaction, CYT: Cell Culture Cytotoxin Neutralization test, EIA: Enzyme Immunoassay.

* To calculate summary statistics, only the first sample was used.

** Excludes a single case of severe CDI where CT was not available at the time