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ORIGINAL PAPER

Toxin positivity and *tcdB* gene load in broad-spectrum *Clostridium difficile* infection

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Abstract

Purpose This study aimed to evaluate the clinical significance of toxin positivity and toxin gene load, and the relation between them in the broad spectrum of *Clostridium difficile* infection (CDI) including colonization, significant diarrhea, and severe disease.

Methods We included 2671 fecal samples submitted for CDI diagnosis and 180 samples from healthy individuals. The clinical spectrum was categorized as category I (toxigenic *C. difficile* positive without clinical CDI criteria), category II (mild CDI), and category III (severe CDI). Clinical parameters were compared based on toxin EIA and *tcdB* C_t values. C_t values of *tcdB* PCR for predicting toxin EIA positivity were assessed using receiver-operating characteristic (ROC) curves.

Results The median C_t values of *tcdB* PCR and toxin positivity were not significantly correlated with clinical spectrum of CDI (27.5, 28.2, and 26.1 for *tcdB* C_t and 55.0, 56.6, and 60.9% for toxin EIA positivity in category I, II, and III, respectively, $P > 0.05$). There were significant differences in the *tcdB* C_t values between toxin EIA-positive and -negative groups ($P < 0.001$). Optimal cutoff for the *tcdB* C_t value for estimating toxin EIA positivity was 26.3 with 79.3% sensitivity and 83.6% specificity with good area under the curves (AUC, 0.848).

Conclusions The C_t values successfully predicted toxin EIA positivity and could be used as a surrogate for toxin EIA positivity in the diagnostic algorithm and routine analysis. Further studies are needed to validate the clinical significance of *tcdB* PCR C_t value in toxigenic *C. difficile* colonization and infection.

Keywords *Clostridium difficile* · Toxin EIA · *tcdB* PCR · Colonization · Cycle threshold

Introduction

Clostridium difficile is a leading cause of antibiotic-associated colitis and imposes a great burden on the healthcare system globally [1, 2]. The clinical manifestations of *C. difficile* infection (CDI) have a broad spectrum ranging from mild diarrhea to toxic megacolon leading to colonic perforation and death. Moreover, they cannot be easily distinguished from other causes including other bacterial or viral infections, use of laxatives, or surgery, which makes an accurate laboratory diagnosis essential [3]. Due to insufficient sensitivity of the toxin enzyme immunoassay (EIA), many laboratories have switched to nucleic acid amplification testing (NAAT) with higher performance [4, 5]. However, use of NAAT as a single test is still questionable in terms of clinical significance and cost-effectiveness [3, 4, 6]. Importantly, *C. difficile* assays cannot distinguish asymptomatic colonization from symptomatic infection, whereas NAAT with its higher sensitivity detects more patients with asymptomatic colonization. Moreover, several studies show that a significant portion of patients with NAAT positive for *C. difficile* did not have clinically significant diarrhea [7, 8]. The experts and guidelines on this issue are still in conflict. The latest European guidelines still recommend the inclusion of toxin EIA in the diagnostic algorithm, whereas other guidelines recommend an NAAT-only approach [9–11]. The rationale of including toxin EIA is based on its correlation with clinical outcomes and CDI-related complications [3, 5]. This study aimed to evaluate the clinical significance of

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toxin positivity and toxin gene load, and the relation between them in the broad spectrum of CDI including colonization, significant CDI diarrhea, and severe CDI.

Methods

Study design and clinical samples

This study was approved by the Institutional Review Board of the Konkuk University Medical Center, Seoul, Korea (a tertiary referral hospital with 900 beds). This study included 2671 patients whose fecal samples were submitted to the clinical microbiology laboratory for *C. difficile* testing from March 2015 to October 2016. As a routine practice of *C. difficile* testing at our hospital, we performed the toxin EIA, and toxigenic culture (TC) and/or *tcdB* PCR. In addition, we included 180 residual stool samples after routine health care examination from asymptomatic healthy individuals and tested them with toxin EIA and *tcdB* PCR. This study required neither study-specific nor any other interventions. Therefore, written informed consent from enrolled patients was exempted.

Laboratory tests

For TC, alcohol-treated stool samples were inoculated onto a chromogenic agar plate (chromID CD agar, bioMérieux, Marcy-l'Etoile, France), and incubated at 37 °C under anaerobic conditions (Forma Anaerobic System; Thermo Fisher Scientific, Waltham, MA, USA). Isolates with typical morphology suspicious for *C. difficile* were confirmed by Gram-staining and finally identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using the VITEK MS system (bioMérieux). Toxin production was confirmed by EIA or in-house PCR which was performed as described previously [12]. Toxin EIA was performed using VIDAS *C. difficile* toxin A&B according to the manufacturer's instructions. The assay principle combined a two-step enzyme immunoassay with fluorescence detection (enzyme-linked fluorescence immunoassay, ELFA) and the results were reported as negative, equivocal, or positive with cutoffs of 0.13 and 0.37 test value (TV) according to the manufacturer's instructions. Equivocal results of toxin EIA were considered negative in this study. The *tcdB* PCR was performed using the Xpert *C. difficile* system (Cepheid, Sunnyvale, CA, USA). This assay detects toxin B (*tcdB*), binary toxin (*cdt*), and a point mutation associated with PCR ribotype 027. Using a maximum valid cycle threshold (C_t) setting of 37 for *tcdB,* the limit-of-detection point estimate for toxigenic *C. difficile* was 1,657 CFUs/swab according to the manufacturer's instructions. We collected the C_t data in all positive results.

Clinical data collection

Among 2671 samples, 366 samples (13.7%) were positive for TC. Among 884 samples tested with *tcdB* PCR, 161 (18.2%) were positive; 434 samples (16.2%) were positive for TC and/or *tcdB* PCR. After excluding 152 duplicated samples in the same episode, we reviewed the data of 282 patients in detail (Fig. 1). For these patients, we collected clinical data through chart review including demographic data and laboratory data including white cell count, serum creatinine, and albumin concentrations tested within 3 days of fecal sample collection. We obtained the baseline serum creatinine concentrations from tests performed more than 6 months before study entry.

The underlying diseases of these patients included malignancy (33.0%), respiratory disease (16.7%), acute infectious diseases (11.0%), cerebral- and cardio-vascular disease (11.0%), surgical disorder (5.3%), and neurologic disease (5.3%). Of the patients, 67.7% had undergone antibiotic therapy and most (95.7%) were treated with metronidazole for CDI. The clinical spectrum was categorized as category I (toxigenic *C. difficile* positive without clinical CDI criteria, < 3 unformed stools in 24 h), mild CDI (≥ 3 unformed stools in 24 h and WBC count less than 15×10^9 cells/L, and serum creatinine less than 1.5 times the premorbid level, category II), and severe CDI (≥ 3 unformed stools in 24 h and WBC count of at least 15×10^9 cells/L or serum creatinine levels at least 1.5 times the premorbid level, category III). CDI-related complications included radiologically confirmed toxic megacolon status or hypovolemic shock with low blood pressure [2, 4]. Patients with laxative use in 48 h before sample collection were excluded from category II and III [13]. The duration of hospitalization and the 30-day all-cause mortalities were assessed as the clinical outcomes. The *tcdB* PCR-positive patients were divided as high C_t

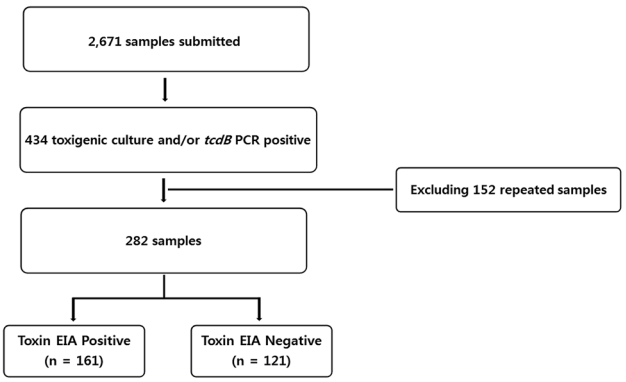

Fig. 1 Distribution of samples according to the detection method for toxigenic *Clostridium difficile*

and low C_t based on the median C_t value (C_t, 27.1). Clinical characteristics were compared between these groups.

Statistical analysis

The difference between the continuous variables was analyzed using the Student's *t* test or Mann–Whitney *U* test, and that between categorical variables was analyzed using the Chi-square test, Fisher's exact test, McNemar test, or the Cochran–Armitage test for trends. The Kruskal–Wallis test and one-way analysis of variance (ANOVA) followed by the Games–Howel's post hoc test was used to assess the differences between groups. The C_t values of *tcdB* PCR for estimating toxin EIA positivity were assessed using receiver-operating characteristic (ROC) curves and the area under the curves (AUC). Statistical analysis was performed using MedCalc Statistical Software (version 15.8, MedCalc Software, Mariakerke, Belgium) and IBM SPSS Statistics 22.0 (IBM Corporation, Armonk, NY, USA). *P* values less than 0.05 were considered statistically significant.

Results

Among 282 patients positive for TC and/or *tcdB* PCR, 119, 99, and 64 patients were category I, II, and III, respectively. The results of toxin EIA and *tcdB* PCR in each clinical category are described in supplemental Table 1. The median C_t values of *tcdB* PCR and toxin positivity were not significantly correlated with clinical spectrum of CDI (27.5, 28.2, and 26.1 for *tcdB* PCR and 55.0, 56.6, and 60.9% for toxin EIA positivity in category I, II, and III, respectively, $P > 0.05$). There were significant differences in age between category II and III (61.5 vs. 67.9 years old, $P = 0.0186$). Comparison of clinical characteristics between groups based on results of the toxin EIA and *tcdB* PCR in patients with TC and/or *tcdB* PCR positivity is described in supplemental Table 2. Age and WBC count were significantly different between groups based on toxin EIA results (mean age, 60.7 vs. 68.0 years old, $P = 0.0016$ and mean WBC count, 8.7 vs. 11.3 10^9/L, $P < 0.0001$, respectively).

The distribution of *tcdB* PCR C_t value between groups divided by toxin EIA results is presented in Fig. 2. There were significant differences in the *tcdB* C_t values between toxin EIA-positive and -negative groups ($P < 0.001$). The optimal cutoff for the *tcdB* C_t value for predicting toxin EIA positivity was established by the ROC curve analysis. The optimal *tcdB* C_t cutoff was 26.3 with 79.3% (95% CI 65.9–89.2) sensitivity, and 83.6% (95% CI 71.2–92.2) specificity with high AUC (0.848, 95% CI 0.767–0.910) (Fig. 3). Among the 180 healthy individuals tested, 8 (4.4%) were positive for toxin EIA and/or *tcdB* PCR. The median C_t value of the *tcdB* PCR-positive healthy group was 32.9 (IQR 30.1–35.2), which was significantly higher than category I, II, and III (C_t, 27.5, 28.2, and 26.1; *P*, 0.0032, 0.0171, and 0.0004, respectively).

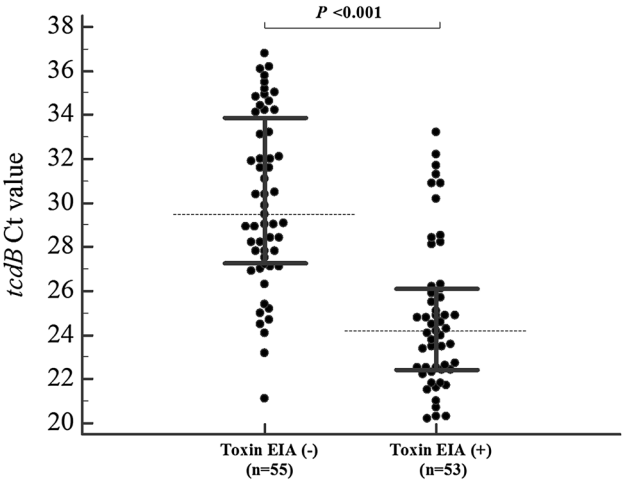

Fig. 2 Distribution of *tcdB* PCR C_t value between groups by toxin EIA results. Black dot line, median value; gray horizontal line, interquartile value

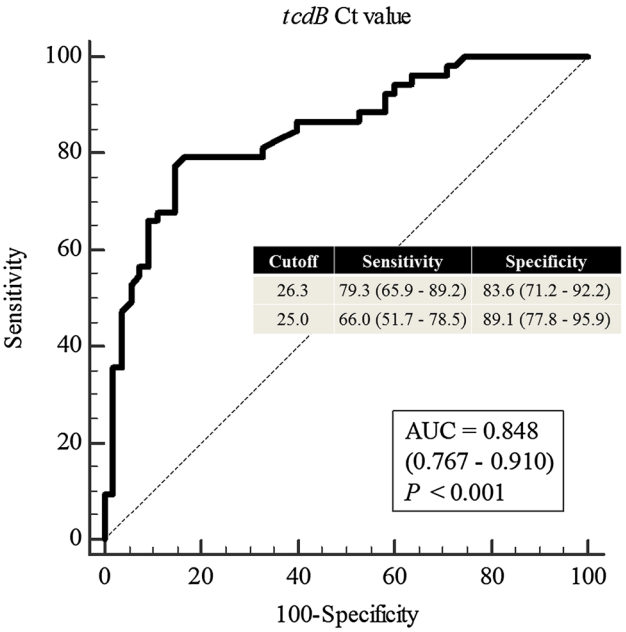

Fig. 3 Receiver-operating curve of *tcdB* cycle threshold (C_t) value for detecting toxin EIA positivity ($n = 108$)

Discussion

In this study, we divided the toxigenic *C. difficile*-positive patients to category I, II, and III adopting the classical clinical criteria. The median C_t values of *tcdB* PCR and rates of

toxin positivity were not significantly correlated with clinical spectrum of CDI (27.5, 28.2, and 26.1 for *tcdB* PCR and 55.0, 56.6, and 60.9% for toxin EIA positivity in category I, II, and III, respectively, $P > 0.05$). Clinical criteria were based on laboratory test and the number of diarrhoeal stools in the medical chart. It can be influenced by the accuracy of medical chart records and there are many factors including drugs, other infections, or host factors that make correct categorization difficult. No reliable laboratory tests accurately distinguish CDI from *C. difficile* colonization and there is also no definite clinical difference between true CDI and non-CDI-related symptoms [9, 14, 15]. Moreover, a very high proportion of laxative therapy in patients diagnosed with CDI has been reported, making clinical criteria based on diarrhoeal stools impractical [7, 13, 16]. We can expect considerable overlap between category I and category II. However, the median C_t value of the *tcdB* PCR in toxigenic *C. difficile*-positive healthy individuals was 32.9, which was significantly higher than those of category I, II, and III. These data suggest significant lower *tcdB* gene load in asymptomatic colonization. We included only 8 toxigenic *C. difficile*-positive individuals among 180 screened subjects. Further study including sufficient number of toxigenic *C. difficile*-positive healthy individuals would be valuable to assess C_t value for differentiation between colonization and significant CDI.

There were significant differences in age between the mild and severe form of CDI (II vs. III) ($P = 0.0186$) (supplemental Table 1). Similarly, there were significant differences for age and WBC count between toxin EIA-negative and -positive groups ($P = 0.0016$ and $P < 0.0001$) (supplemental Table 2). The WBC count might be related to older age in toxin positive groups, suggesting more severe clinical findings in older groups. Regarding clinical impact of toxin positivity, there are different results between studies and more controlled study is needed to elucidate this issue [3, 8, 17].

Currently, NAAT has become the predominant method for CDI diagnosis and some experts prefer an NAAT-only approach [10, 11, 18]. Nevertheless, recent studies have shown the clinical importance of toxin positivity [3, 5] and toxin EIA is still recommended in updated guidelines as the second step, a highly specific test in a two-step algorithm [9]. Positive results of toxin EIA have two meanings including positive free toxin in feces and organism burden above the threshold of toxin EIA [9]. As the sensitivity of toxin EIA is lower than that of NAAT, the clinical correlation of positive toxin EIA could reflect the impact of higher organism burden compared to that of positive NAAT. The C_t value of real-time PCR or DNA copies can also reflect the organism burden and has shown a correlation with toxin EIA [5, 19]. Some recent studies evaluated the clinical impact of C_t value in CDI [20, 21]. In this study, there was a significant difference in the C_t value between toxin EIA-negative and -positive groups (median 29.5 vs 24.2, $P < 0.001$). Using the ROC curve analysis of C_t value for detecting toxin EIA positivity, a good AUC value of 0.848 was obtained with the optimal C_t cutoff (26.3). Using an algorithm with additional toxin, EIA steps could require additional time, cost for the additional step, and result in diagnostic delay. Based on our results, it could be replaced by using C_t values with no additional cost. The optimal cutoff from ROC analysis is close to median C_t value of all positive results (C_t, 26.3 vs. 27.1), and could be applied to each laboratory. This cutoff is also very close to that of recent study on this issue (26.35), indicating easy adaptation to other laboratory [22]. All C_t values of *tcdB* PCR-positive results in healthy individuals have shown an above optimal cutoff (26.3).

This study has its strengths and limitations. We analyzed the clinical values of toxin EIA positivity and *tcdB* gene load PCR in the broad spectrum of CDI ranging from healthy carriers to severe CDI. In addition, based on our results and published studies, it could be possible to adopt the C_t value instead of the toxin EIA into the algorithm and routine analysis. The limitation of this study is the difference in the number of tested patients between toxin EIA and *tcdB* PCR. All patients were tested with TC and toxin EIA, but only 33% were tested with *tcdB* PCR. The lower number of *tcdB* PCR positivity compared to toxin EIA positivity can affect the statistical significance of the parameters between the low and high C_t groups. Further studies are needed to validate the values of C_t of *tcdB* PCR.

In conclusion, the C_t values successfully predicted toxin EIA positivity, and could be used as a surrogate for toxin EIA positivity in the diagnostic algorithm and routine analysis. Further studies are needed to validate the clinical significance of *tcdB* PCR C_t value in toxigenic *C. difficile* colonization and infection.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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