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MAJOR ARTICLE

Binary Toxin Expression by *Clostridioides difficile* Is Associated With Worse Disease

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Background. The incidence of *Clostridioides difficile* infection (CDI) has increased over the past 2 decades and is considered an urgent threat by the Centers for Disease Control and Prevention. Hypervirulent strains such as ribotype 027, which possess genes for the additional toxin *C. difficile* binary toxin (CDT), are contributing to increased morbidity and mortality.

Methods. We retrospectively tested stool from 215 CDI patients for CDT by enzyme-linked immunosorbent assay (ELISA). Stratifying patients by CDT status, we assessed if disease severity and clinical outcomes correlated with CDT positivity. Additionally, we completed quantitative PCR (PCR) DNA extracted from patient stool to detect cdtB gene. Lastly, we performed 16 S rRNA gene sequencing to examine if CDT-positive samples had an altered fecal microbiota.

Results. We found that patients with CdtB, the pore-forming component of CDT, detected in their stool by ELISA, were more likely to have severe disease with higher 90-day mortality. CDT-positive patients also had higher *C. difficile* bacterial burden and white blood cell counts. There was no significant difference in gut microbiome diversity between CDT-positive and -negative patients.

Conclusions. Patients with fecal samples that were positive for CDT had increased disease severity and worse clinical outcomes. Utilization of PCR and testing for *C. difficile* toxins A and B may not reveal the entire picture when diagnosing CDI; detection of CDT-expressing strains is valuable in identifying patients at risk of more severe disease.

Keywords. *C. difficile*; CDT; binary toxin; CDI; ribotype 027.

Clostridioides difficile (*C. difficile*) is a gram-positive, spore-forming bacterium that can cause diarrhea and colitis in patients with dysbiosis. In 2017, *C. difficile* infection (CDI) affected >200 000 people per year, resulting in almost 13 000 deaths, and was considered an urgent antibiotic-resistant threat by the Centers for Disease Control and Prevention [1]. The increased prevalence of CDI is attributed to novel hypervirulent strains that cause worse disease and higher mortality [2]. In addition to the main virulence factors, toxins A and B, these hypervirulent strains produce a third toxin known as *C. difficile* binary toxin (CDT) or binary toxin. CDT is comprised of CdtA, an actin-specific ADP-ribosyl transferase, and CdtB, the receptor-binding component [3].

With the increased prevalence of CDI in the United States, there has been substantial debate over the optimal diagnostic approach [4]. The use of highly sensitive polymerase chain reaction (PCR; which detects the presence of the *C. difficile* toxin gene but not protein) and increased clinical testing of asymptomatic individuals are believed to contribute to the overdiagnosis of CDI [5–7]. PCR-positive, toxin A/B enzyme immunoassay (EIA)–positive patients have increased disease severity compared with patients with discordant tests (PCR+, toxin A/B EIA-) [7–9]. Previous studies have shown that detection of CDT genes in stool by PCR is associated with worse outcomes, but no investigation has been done to compare disease severity by immunoassay [2, 10–12].

In this study, we wished to further explore the relationship between CDT and disease severity by testing for toxin in stool with enzyme-linked immunosorbent assay (ELISA). We found that CDI patients with CDT-expressing strains had increased disease severity and worse clinical outcomes.

METHODS

Sample Collection and Patient Information

Stool samples collected from 215 CDI patients were retrospectively identified from the University of Virginia (UVA) Medical Center's electronic medical records (EMR). Stool samples were collected between 2015 and 2017 and were held in the clinical laboratory at 4°C for 48 hours until considered "discarded,"

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then aliquoted and stored at −80°C until testing. All patients had diarrhea and were positive for *C. difficile* TcdB by Xpert *C. difficile* PCR (Cepheid, Sunnyvale, CA, USA) at the University of Virginia (UVA) Clinical Microbiology Laboratory. Data on the presence or absence of CDT genes were not collected, nor were isolates collected for typing. Patient demographics, clinical lab results, and disease outcomes were collected retrospectively from the UVA Clinical Data Repository and the EMR. The collection of patient data was approved by the institutional review board (protocol IRB-HSR 16926).

CdtB Detection

Stool samples from 215 patients were tested using a previously described research-only ELISA for *Clostridium difficile* cdtB [13].

C. difficile TcdA and TcdB Detection by ELISA

All stool samples were tested for TcdA and TcdB by *C. DIFFICILE* TOX A/B II according to the manufacturer's instructions (catalog No. T5015, TechLab Inc, Blacksburg, VA, USA).

Lactoferrin Detection

Stool lactoferrin was assayed using LACTOFERRIN SCAN according to manufacturer instructions (catalog No. T5009/30351, TechLab Inc, Blacksburg, VA, USA).

Fecal DNA Extraction/16S rRNA Gene V4 Region Sequencing

DNA from patient fecal samples was extracted using the QIAamp DNA Stool Mini Kit (catalog No. 51504, Qiagen, Hilden, Germany). For each sample, the V4 region of the 16S rRNA gene was amplified using the dual indexing sequencing strategy, as described previously [14]. Sequencing was done on the Illumina MiSeq platform using a MiSeq Reagent Kit, version 2 (500 cycles; catalog No. MS102-2003, Illumina Inc, San Diego, CA, USA), according to the manufacturer's instructions, with modifications found in the Schloss SOP: https://github.com/SchlossLab/MiSeq_WetLab_SOP. The mock community ZymoBIOMICS Microbial Community DNA Standard (catalog No. D6306, Zymo Research, Irvine, CA, USA) was sequenced to monitor sequencing error.

16 S rRNA Gene Amplicon Curation and Analysis

The 16S data curation and analysis from the human stool samples were performed using R, version 4.0.3. Sequences were curated using the R package DADA2, version 1.18 [15]. Briefly, reads were filtered and trimmed using standard parameters outlined in the DADA2, version 1.8, pipeline. The error rates for the amplicon data sets were determined using DADA2's implementation of a parametric error model. Samples were then dereplicated, and sequence and variants were inferred. Overlapping forward and reverse reads were merged, and sequences that were <251 bp or >255 bp were removed. Finally, chimeras were removed. Taxonomy was assigned to amplicon sequence variants (ASVs) using the DADA2-formatted SILVA taxonomic training data release 132 [16]. Samples with <16 000 reads per sample were removed from the analysis (this included the extraction blank). The sequences associated with this analysis will be deposited in the SRA. Following sequence curation, the packages phyloseq, version 1.34.0, vegan, version 2.5.7, dplyer, version 1.0.6, and ggpubr, version 0.4.0, were used for analysis and generation of figures [17].

Quantitative Real-time PCR for the CdtB Gene

PCR was performed for the identification of the CDT-expressing gene, cdtB. Primers and probe sequences used have been previously described by Wroblewski et al. [18]. The PCR reaction volume of 20 µL included 2 µL of stool DNA, 6 µM of each primer, 4 µM of the FAM-BHQ1 probe, and 10 µL of iQ Multiplex Powermix (catalog No. 1725849, Bio-Rad Laboratory, Inc., Hercules, CA, USA). Reactions were initiated at 95°C for 3 minutes, followed by 40 cycles of 95°C for 20 seconds, 61°C for 40 seconds, 72°C for 40 seconds, and a final step at 72°C for 5 minutes in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratory, Inc., Hercules, CA, USA). The Ct cutoff value was 35. Sanger sequencing was completed on 2 PCR products to ensure proper amplification. Additionally, as a control for fecal DNA quantity, quantitative PCR (qPCR) was used to detect Human RNase P following the manufacturer's protocol, modified using iQ Multiplex Powermix (catalog No. 2019-nCoVEUA-01) [19]. Any DNA samples with insufficient DNA by RNase P qPCR were excluded from all comparisons.

Statistical Methods

All statistical comparisons and graphs were made using GraphPad Prism 9 or using R, version 4.0.3. Comparisons of CDT ELISA/PCR–positive and CDT ELISA/PCR–negative patient demographics, toxin A/B status, and disease outcomes were calculated using the chi-square test. Differences in white blood cell (WBC) counts, clinical TcdB PCR cycle threshold (Ct) values, stool lactoferrin, days in the intensive care unit (ICU), and days hospitalized between CDT ELISA–positive and CDT ELISA–negative patients were calculated using a Mann-Whitney *U* test. Differences between the Simpson and Shannon indexes were found using a Wilcoxon signed-rank test. A *P* value >.05 was considered significant.

RESULTS

Patient Characteristics and Clinical Outcomes

Of the 215 patient stool samples analyzed, 32 were ELISA-positive for CDT protein (Table 1). Both the CDT ELISA–positive and CDT ELISA–negative cohorts were split evenly by sex, and there was no significant difference in age ($P = .1$). CDT ELISA–positive patients were more likely to be positive by ELISA for toxins A/B ($P = .0002$) (Table 1). Patients

Table 1. Patient Characteristics and Disease Outcomes

	CDT ELISA+ (n = 32)	CDT ELISA- (n = 182)	*P* Value
Age, mean (SD),[a] y	64.7 (+/-16.2)	59.0 (+/-17.0)	.10
Sex, No. (%)[a]			.93
Female	16 (50)	85 (50.1)	
Toxin A/B ELISA+, No. (%)	24 (75)[b]	71 (39.4)	**.0002**
WBC >15 000/μL, No. (%)[a]	14 (45.2)	34 (19.5)	**.002**
Admitted to ICU, No. (%)[a]	11 (34.4)	28 (15.6)	**.01**
Deceased within 90 d of diagnosis, No. (%)[a]	7 (23.3)	16 (9.6)	**.03**

Bolded values considered statistically significant ($P < .05$).

Abbreviations: CDT, *Clostridioides difficile* toxin; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; WBC, white blood cell count.

[a]Some data missing, percentage based on patients with data available.

[b]Four of the 8 CDT+/toxin A/B- samples were close to the CDT+ optical density (OD) cutoff.

testing positive for CDT by ELISA were also more likely to have a WBC >15 000/μL ($P = .002$), to be admitted to the ICU ($P = .01$), and to have died by 90 days after diagnosis ($P = .03$).

Patients Testing Positive for CDT by ELISA Had Increased *C. difficile* Bacterial Burden and White Blood Cell Count

The 32 CDT ELISA–positive patients had significantly higher WBC ($P = .01$) (Figure 1A). These patients also had a higher *C. difficile* bacterial burden, as indicated by a lower Ct value for the toxin B gene ($P = .001$) (Figure 1B). When we compared bacterial burden from the samples that were discordant by ELISA (ie, CDT ELISA–positive, –negative toxin A/B ELISA) with the burden in the 24 patients who tested positive by ELISA for both CDT and toxins A/B, those who were positive for all toxins had significantly lower TcdB Ct values ($P = .01$) (Supplementary Figure 1). Thus, patients with CDT ELISA–positive, toxin A/B–positive samples had higher *C. difficile* burden compared with patients who were ELISA-positive for CDT and negative for toxin A/B. Patients testing ELISA-positive for CDT had a nonsignificantly higher lactoferrin level compared with CDT ELISA–negative patients ($P = .06$) (Figure 1C).

CDT ELISA–Positive Patients Had Increased Hospital and ICU Stays

CDT ELISA–positive patients had longer hospitalizations ($P = .05$) and days in the ICU ($P = .008$) (Figure 2). Patients testing positive for CDT by ELISA stayed in the hospital 6.5 days longer than CDT ELISA–negative patients. CDT ELISA–negative patients stayed in the ICU on average 1.7 days compared with 3.6 days for CDT ELISA–positive patients.

Disease Outcomes of CDT qPCR–Positive Patients

Of the patients in this cohort, 70 had sufficient residual stool DNA to be tested by qPCR for the CDT gene. Sixteen were qPCR-positive for cdtB. Patients positive by qPCR for cdtB were more likely to test positive for toxins A/B by ELISA compared with cdtB-negative patients ($P = .02$) and those with WBC >15 000/μL ($P = .06$). There were too few patients tested by qPCR for cdtB to draw conclusions about ICU admission and 90-day mortality (Supplementary Table 1).

No Differences in Gut Microbiome Alpha Diversity Between CDT ELISA–Positive and –Negative Patients

We sought to determine if the structure of the fecal microbiome was altered in CDT ELISA–positive patients. Composition

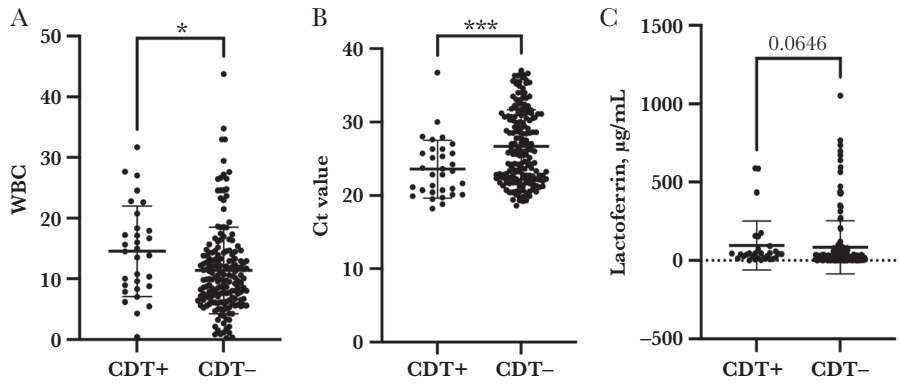

Figure 1. Patients testing positive for CDT by ELISA had increased bacterial burden, white blood cell count, and intestinal inflammation. A, Patients with CDT-expressing strains had higher white blood cell counts than patients with non-CDT-expressing strains ($P = .01$). B, CDT-positive patients had lower Ct values \than CDT-negative patients, indicating increased bacterial burden ($P = .001$). C, CDT-positive patients had elevated lactoferrin compared with CDT-negative patients, although the level was not statistically significant ($P = .06$). Abbreviations: CDT, *Clostridioides difficile* toxin; Ct, cycle threshold; ELISA, enzyme-linked immunosorbent assay; WBC, white blood cell count.

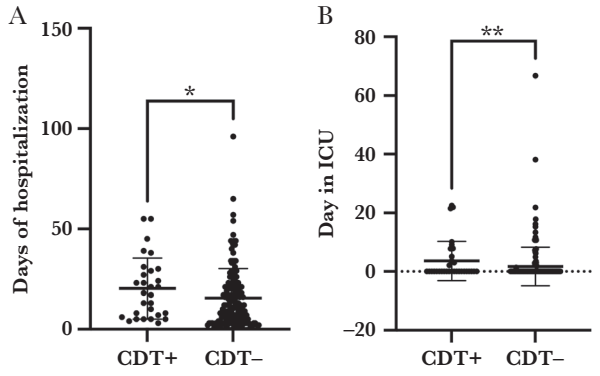

Figure 2. CDT ELISA–positive patients had increased hospital and ICU stays. A, Patients with CDT detected in their stool had longer stays in the hospital, with an average stay of 18 days compared with 11.5 days for CDT-negative patients. ($P = .05$). B, CDT-positive patients had longer stays in the ICU ($P = .01$). Abbreviations: CDT, *Clostridioides difficile* toxin; Ct, cycle threshold; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit.

of the fecal microbiota from 172 patients was profiled using amplicon sequencing of the 16S rRNA gene V4 region. While CDT ELISA–negative patients trended toward slightly increased alpha diversity as measured by either Simpson diversity index or Shannon diversity, there was not a significant difference using either metric ($P = .26$, $P = .16$) (Figure 3A, B).

DISCUSSION

Previous studies have shown that infection with strains positive for the cdtB gene result in increased disease severity, increased disease recurrence, and higher mortality rates [2, 10–12]. Here we further show that patients with CDT protein detected by ELISA in stool had worse disease outcomes, measured by increased rates of ICU admissions and higher mortality. Additionally, these patients had longer stays in the hospital and ICU, causing increased burden to health care systems. Other measures of disease severity were also worse in CDT ELISA–positive infections, including an elevated white blood cell count >15 000/μL as previously observed by PCR detection of CDT [2, 10, 11]. Patients with CDT ELISA–positive stools also exhibited higher *C. difficile* bacterial burdens as indicated by lower Ct values, consistent with higher burden being correlated with more severe disease [12, 20]. We confirmed the earlier finding that CDT ELISA–positive patients had higher lactoferrin levels, although in this case our findings were not statistically significant [21].

Lastly, in contrast to studies that have described a decreased gut microbiome diversity in patients with CDI compared with control patients [22–24], we found no significant differences in Simpson or Shannon indexes when comparing CDT+ vs CDT- *C. difficile* infections. These results indicate that increased disease severity related to CDT may not be associated with decreased gut microbiome diversity.

In conclusion, patients with stool testing positive for CDT by ELISA had more severe CDI and worse disease outcomes. The presence of stool CDT protein was associated with higher *C. difficile* bacterial burden and WBC counts, as well as longer hospital and ICU stays and increased mortality. Therefore, characterizing *C. difficile* infection with CDT-expressing strains may be beneficial in clinical disease diagnosis and treatment.

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Potential conflicts of interest. W.A.P. is a consultant for TechLab Inc., and D.M.L., R.J.C., and M.W.L. are TechLab Inc employees. All other authors declare no conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

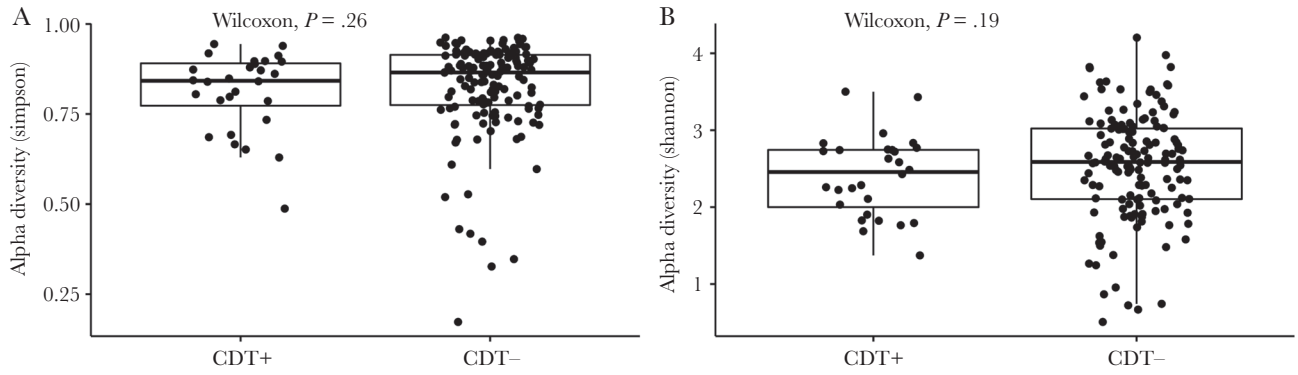

Figure 3. No differences in gut microbiome diversity between CDT-positive and -negative patients. A, There was no significant difference in Simpson Diversity Index between CDT-positive and -negative patients ($P = .26$). B, Shannon Diversity Index was slightly higher in CDT-negative patients, although not statistically significant so ($P = .16$). Abbreviation: CDT, *Clostridioides difficile* toxin.

Ethics approval and patient consent. The collection of deidentified patient samples and information was reviewed and approved by the UVA IRB (protocol IRB-HSR 16926) in compliance with federal regulations protecting human participants.

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