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Absence of Toxemia in *Clostridioides difficile* infection: Results from Ultrasensitive Toxin Assay of Serum

Rebecca Sprague[1], **Karolyne Warny**[1], **Nira Pollock, MD, PhD**[2,3], **Kaitlyn Daugherty**[1], **Qianyun Lin, MD**[1], **Hua Xu**[1], **Christine Cuddemi**[1], **Caitlin Barrett**[1], **Xinhua Chen, PhD**[1], **Alice Banz, PhD**[4], **Aude Lantz**[4], **Kevin W. Garey, PharmD, MS**[5], **Anne J. Gonzales-Luna, PharmD, BCIDP**[5], **Carolyn D. Alonso, MD**[2], **Javier A. Villafuerte Galvez, MD**[1], **Ciarán P. Kelly, MD**[1]

[1]Divisions of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center & Harvard Medical School, 330 Brookline Avenue, Boston, Massachusetts, 02215 USA.

[2]Divisions of Infectious Disease, Department of Medicine, Beth Israel Deaconess Medical Center & Harvard Medical School, 330 Brookline Avenue, Boston, Massachusetts, 02215 USA.

[3]Department of Laboratory Medicine, Boston Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts, 02215 USA.

[4]BioMérieux, 100 Rue Louis Pasteur, 69280, Marcy-l'Étoile, France.

[5]Department of Pharmacy Practice and Translational Research, University of Houston College of Pharmacy, 4849 Calhoun Rd #3044, Houston, Texas, 77204 USA.

Abstract

Clostridioides difficile infection (CDI) is caused by Toxins A and B, secreted from pathogenic strains of *C.difficile*. This infection can vary greatly in symptom severity and in clinical presentation. Current assays used to diagnose CDI may lack the required sensitivity to detect the exotoxins circulating in blood. The ultrasensitive Single Molecule Array (Simoa) assay was modified to separately detect toxin A and toxin B in serum with a limit of detection at the low picogram level. When applied to a diverse cohort, Simoa was unable to detect Toxin A or B in serum from patients with CDI, including many classified as having severe disease. The detection of toxin may be limited by the inferenece of anti-toxin anti-bodies circulating in serum. This result does not support the hypothesis that toxemia occurs in *C. difficile* infection, conflicting with the findings of other published reports.

Keywords

Clostridioides difficile ; Simoa ultrasensitive toxin assay; antibiotic-associated diarrhea; infectious diarrhea; nosocomial infection; colitis

Corresponding Author: Rebecca Sprague, Beth Israel Deaconess Medical Center, Division of Gastroenterology, Dana 617D, 330 Brookline Avenue, Boston, MA 02215, Phone: (617) 667-3419, Fax: (617) 667-8838, rsprague@bidmc.harvard.edu.

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Introduction

Clostridioides difficile infection (CDI) is the major cause of hospital-acquired bacterial infectious diarrhea with disease manifestations that range from mild diarrhea to severe pseudomembranous colitis, resulting in colectomy or even death [1,2]. CDI is estimated to cause 29,000 deaths annually in the United States [3], and the rate of severe clinical presentation and negative clinical outcomes continues to increase [1]. Despite the increased prevalence of CDI, there is not a consensus among the international medical community regarding the optimal method of detection and diagnosis [4].

Diarrhea and colitis in CDI are caused by Toxin A (TcdA) and Toxin B (TcdB), two protein exotoxins secreted by pathogenic strains of *C. difficile* [5]. In fulminant CDI, these toxins lead to systemic complications and extra-colonic manifestations consistent with sepsis or even toxemia, including multiple organ dysfunction [6]. However, identification of toxemia in CDI patients is incredibly rare, with two reported cases out of seven children studied [7], and two reported cases out of eighty-eight adults studied [8].

We hypothesized that the rarity at which toxemia is detected in CDI may be due to very low concentrations of circulating toxin in the blood, below the limit of detection of commercially available toxin assays. Standard enzyme immunoassay (EIA) has a limit of detection (LOD) of ~1000 pg/ml, while the tissue culture cytotoxicity assay has a LOD of ~100 pg/ml [9]. Previously, our group helped to develop ultrasensitive assays for the detection of TcdA and TcdB in stool using Single Molecule Array (Simoa) [9,10]. Here, we modified the Simoa toxin assays for the detection of toxins in serum and applied the assay to a large panel of serum samples from patients with confirmed CDI.

Methods

Enrolled subjects were inpatients at Beth Israel Deaconess Medical Center (Boston, MA) or Texas Medical Center (Houston, TX) between June 2016 and February 2018. Eligible patients were ≥ 18 years old with a positive clinical *C. difficile* nucleic acid amplification test (NAAT) result in stool, had initiated CDI therapy, and had acute diarrhea. Acute diarrhea was defined by three or more unformed bowel movements during a 24 hour period, persistent diarrhea reported in physician or nursing progress notes, pseudomembranous colitis, or chronic diarrhea with an acute increase.

Patients were excluded if their baseline stool sample was ≥ 72 hours old, or if they had received > 48 hours of CDI treatment. Discarded baseline stool and serum (latter drawn within 1 day of the stool sample) samples were captured. Serum samples were promptly stored at 4°C, aliquoted within 5 days, and frozen at −80°C. A severe CDI outcome was defined as ICU admission, colectomy, or death within 40 days of CDI diagnosis. The attribution of these severe outcomes and classification of disease severity was as previously described [11].

Toxin Simoa assays were performed on a Simoa HD-1 Analyzer (Quanterix Corporation, Lexington, MA) and utilized paramagnetic beads coated with a monoclonal antibody (mAb) directed against TcdA or TcdB (bioMérieux, Marcy L'Etoile) and a detector mAb (against

TcdA or TcdB) conjugated to β-galactosidase (Roche Laboratories, Cambridge, MA). Native toxins A and B (Native Antigen Company, Oxfordshire, UK) were used as standards. The Simoa limit of detection (LOD) for toxins in serum was defined using sera (containing no anti-toxin antibodies, as defined by ELISA) spiked with native toxins [12]; LODs were 10.9 pg/ml for TcdA and 15.7 pg/ml for TcdB. Clinical cut-offs were defined by signal (mean plus two standard deviations) observed in sera from patients without CDI or *C. difficile* carriage, and were 15.0 pg/ml for TcdA and 26.7 pg/ml for TcdB. Clinical serum samples were tested in duplicate with toxin standards and two positive control samples (sera spiked with TcdA or TcdB).

Results

Our cohort included 169 patients with a median age of 68 years (IQR 54–78), most with severe CDI and many with severe clinical outcomes attributed to CDI including ICU admission, colectomy or death (Table 1). Based on the presumed correlation between severe infection and higher levels of circulating toxin, in combination with this ultrasensitive toxin assay, detectable toxin levels were expected. However, we found no detectable TcdA or TcdB in the serum of our patient cohort despite a wide range of toxin concentrations in paired stool (Figure 1).

In 10.6% of serum samples, an artifact was observed in which a small percentage of beads demonstrated many bound antibody complexes with high enzymatic activity consistent with background signal related to binding of an unknown interfering molecule. Additional testing was performed to evaluate the sera with the highest signal readings below the clinical cut-offs. These other test methods included Western Blotting using toxin antibodies, Mass Spectrometry, and Tissue Culture Cytotoxicity Assay. All of these tests yielded negative results for the toxins.

Circulating anti-toxin antibodies in blood may interfere with TcdA and/or TcdB detection. Serum samples with varying amounts of IgA, IgG or IgM anti-toxin (measured by ELISA) were spiked with varying amounts of TcdA or TcdB and toxin concentrations were then measured by Simoa. High anti-toxin antibody concentrations were associated with loss of Simoa signal, suggesting substantial inhibition of toxin measurements. There was also a weak negative but statistically significant correlation between stool TcdB levels and serum anti-toxin B IgA and IgG levels (rho=−0.26, rho=−0.25, respectively; P=0.0001 for both), making plausible the hypothesis that anti-toxin antibodies contribute to the absence of detectable serum toxin by Simoa. Of note, however, anti-toxin antibodies are also present in stool where toxins are routinely detected by substantially less sensitive EIAs.

Discussion

In contrast to earlier published findings which reported on the presence of detectable toxin in the serum of patients with CDI, our work did not support this observation. There were several limitations of this study potentially contributing to these negative results. Serum samples for this study were refrigerated, frozen, and then thawed before testing which could have caused toxin degradation; however, stool toxin measurements were made in stool

refrigerated for up to 72h prior to freezing, and after single thaw. In addition, as fulminant findings related to CDI are rare, only 2 fatal outcomes are included in our study population. Though the Simoa assay is ultrasensitive, it is plausible that the circulating toxin levels still remain below its limit of detection. Alternative assays were used in previous studies where toxemia was reported. The first study used a tissue culture cytotoxicity assay (LOD ~1 – 10 ng/mL for TcdA and ~10 to 100 pg/mL for TcdB.[7] The second study used an ultrasensitive immunocytotoxicity (ICT) assay with a LOD of 0.1 to 1.0 pg/ml for TcdA (when used with enhancing antibody A1H3) and 10.0 pg/ml for TcdB [8]. It is also possible that other components circulating in serum bind to the toxins, preventing detection.

Although Simoa is highly sensitive for detection of picogram quantities of TcdA or TcdB it was unable to detect either toxin in serum during CDI. In some instances detection may have been inhibited by anti-toxin antibodies. However, our study findings do not support the hypothesis that toxemia develops even in severe CDI.

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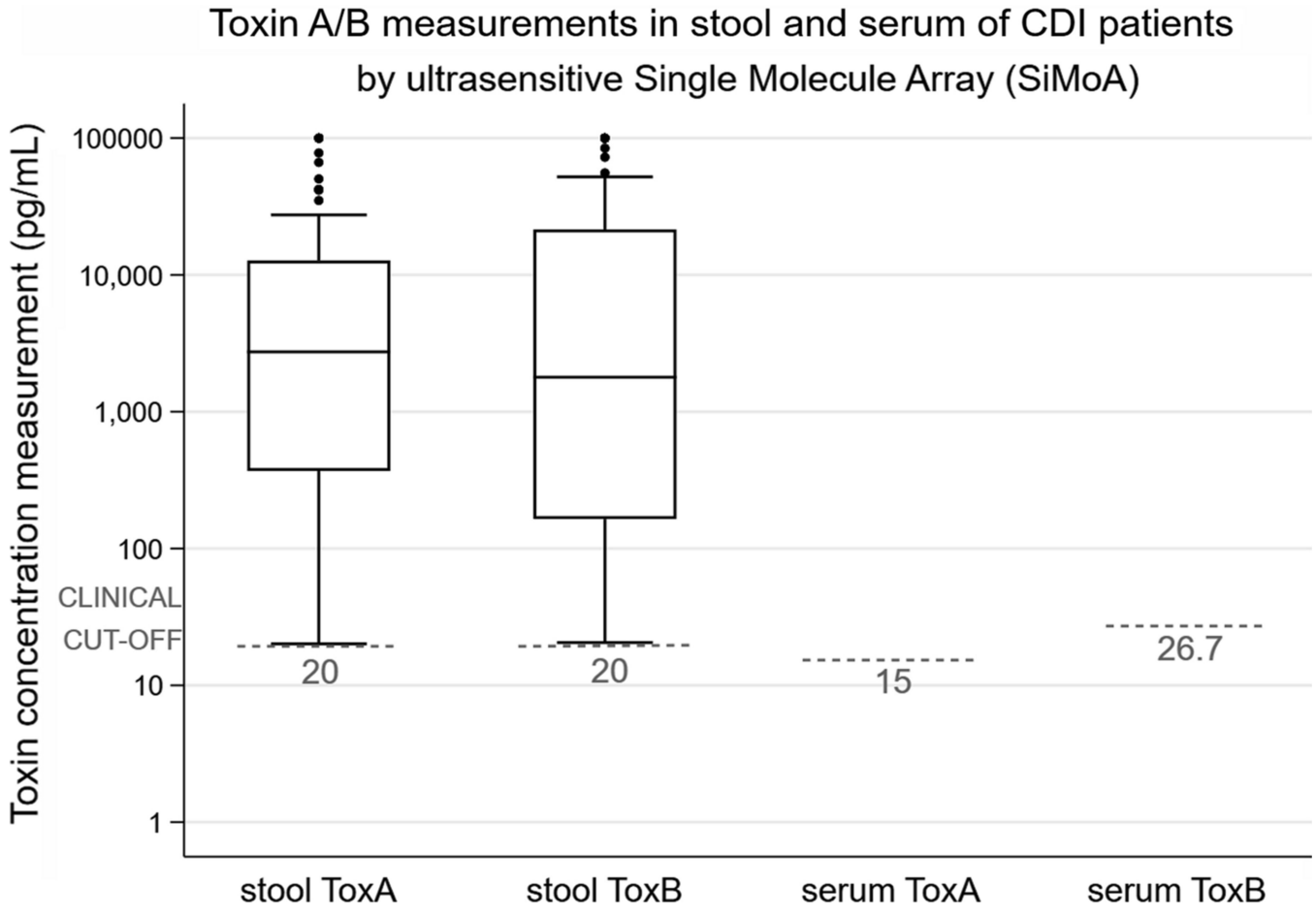

Figure 1.

Comparison of TcdA and TcdB concentrations, as measured by Simoa, in serum and stool. Clinical cutoffs are shown: stool, 20 pg/ml for TcdA and for TcdB; serum 15.0 pg/ml for TcdA and is 26.7 pg/ml for TcdB. Signals below these cutoffs are considered background (negative).

Table 1.

Demographics, Baseline Laboratory Values, and Clinical Outcomes for the cohort

Variable	n = 169	
Demographic Information		
Age Median (IQR)	68 (54 – 78)	
Male Gender	90	53.3%
Race		
White	126	74.6%
African American	21	12.4%
Other	18	10.7%
Unknown	4	2.4%
Ethnicity		
Hispanic	9	5.3%
Not Hispanic	144	85.2%
Unknown	16	9.5%
Laboratory Results		
WBC (10^3 cells/μL) Median (IQR)	11.5 (7.4 – 18.6)	
WBC ≥15 × 10^3 cells/μL	61	36.1%
Creatinine (mg/dl) Median (IQR)	1.1 (0.8 – 1.9)	
Creatinine ≥ 1.5 mg/dl	61	36.1%
Albumin (g/dl) Median (IQR)	3 (2.5 – 3.6) n = 152	
Albumin ≤ 3 g/dl	73 (n = 152)	43.2%
027 / NAP1 / B1 strain	17	10.1%
Severe Clinical Outcomes - Total		
ICU admission	24	14.2%
Colectomy	1	0.6%
Death within 40 days	14	8.3%
Severe Clinical Outcomes – Attributed to CDI		
ICU admission	13	7.7%
Colectomy	1	0.6%
Death within 40 days	2	2.4%
Severity Classifications* n = 153		
IDSA Severe	90	74.4%
ESCMID Severe	93	76.9%
Zar *et al* Severe	73	60.3%
Belmares *et al* Severe	23	19.0%

For explanations of each severity classification with sources see reference 11