Clinical Infectious Diseases

MAJOR ARTICLE

Toxin A–Predominant Pathogenic *Clostridioides difficile*: A Novel Clinical Phenotype

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Background. Most *Clostridioides difficile* toxinogenic strains produce both toxins A and B (A^+B^+), but toxin A–negative, toxin B–positive (A^-B^+) variants also cause disease. We report the identification of a series of pathogenic clinical *C. difficile* isolates that produce high amounts of toxin A with low or nondetectable toxin B.

Methods. An ultrasensitive, quantitative immunoassay was used to measure toxins A and B in stool samples from 187 *C. difficile* infection (CDI) patients and 44 carriers. Isolates were cultured and assessed for in vitro toxin production and in vivo phenotypes (mouse CDI model).

Results. There were 7 CDI patients and 6 carriers who had stools with detectable toxin A (TcdA, range 23–17 422 pg/mL; 5.6% of samples overall) but toxin B (TcdB) below the clinical detection limit (<20 pg/mL; median TcdA:B ratio 17.93). Concentrations of toxin A far exceeded B in in vitro cultures of all 12 recovered isolates (median TcdA:B ratio 26). Of 8 toxin A>>B isolates tested in mice, 4 caused diarrhea, and 3 of those 4 caused lethal disease. Ribotyping demonstrated strain diversity. TcdA-predominant samples were also identified at 2 other centers, with similar frequencies (7.5% and 6.8%).

Conclusions. We report the discovery of clinical pathogenic *C. difficile* strains that produce high levels of toxin A but minimal or no toxin B. This pattern of toxin production is not rare (>5% of isolates) and is consistently observed in vitro and in vivo in humans and mice. Our study highlights the significance of toxin A in human CDI pathogenesis and has important implications for CDI diagnosis, treatment, and vaccine development.

Keywords. *C. difficile*; toxin A; toxin B.

Clostridioides difficile is an anaerobic, spore-forming, gram-positive bacterium and is the most prevalent cause of infectious diarrhea and pseudomembranous colitis in hospitalized patients. Despite significant advances in the field of *C. difficile* infection (CDI) over the past 3 decades, CDI remains responsible for high morbidity and mortality [1, 2]. The major virulence factors of *C. difficile* are thought to be 2 structurally similar toxins, denoted as toxin A and toxin B (TcdA and TcdB, encoded by the *tcdA* and *tcdB* genes) [3]. Prior to the 1990s, pathogenic strains were thought to always simultaneously produce both toxins (A^+B^+). However, with the development of new detection technologies and the potential evolution of *C. difficile*, numerous toxin A–negative, toxin B–positive (A^-B^+) strains have been isolated worldwide since the early 1990s [4, 5]. These isolates cause the full disease spectrum of CDI, despite only producing 1 of the 2 major toxins [5, 6]. The increased frequency of A^-B^+ isolates and their demonstrated ability to cause severe disease [5] have triggered many investigations that have highlighted the importance of toxin B in the pathogenesis of CDI and downplayed the significance of toxin A. Although both toxin A and B can independently cause disease in animal models [3, 7], toxin B is now often considered the primary mediator of symptomatic infection, leading to a focus on toxin B as a diagnostic and therapeutic target.

Reports of naturally occurring toxin B–deficient mutant isolates (A^+B^- variants) are extremely rare. We found 1 case report from 1998 of infection with a suspected A^+B^- strain [8, 9]. However, this strain did not produce any detectable toxin A in vitro, bringing into question its relevance as the cause of the patient's symptoms. Another sporadic case of apparent infection caused by an A^+B^- strain has been described, and the toxin B deficiency was confirmed by cytotoxicity assay and DNA sequencing [10]. Importantly, however, A^+B^- variants have not previously been convincingly demonstrated to cause human CDI.

Received 9 April 2019; editorial decision 22 July 2019; accepted 30 July 2019; published online August 11, 2019.

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Clinical Infectious Diseases® 2020;70(12):2628–33

DOI: 10.1093/cid/ciz727

We here report the identification of a series of pathogenic clinical *C. difficile* strains that produced high amounts of toxin A with low or nondetectable toxin B, demonstrating that toxin A–predominant strains can also cause human CDI.

MATERIALS AND METHODS

Clinical Stool Specimens and Isolation of Bacterial Strains

Stool samples were collected from 187 CDI patients and 44 carriers at Beth Israel Deaconess Medical Center under Internal Review Board–approved protocols, as previously described [11, 12]; all samples tested positive by the Cepheid Xpert *C. difficile*/Epi nucleic acid amplification test (NAAT; Cepheid, Sunnyvale, CA), which detects the presence of the *tcdB* gene. Briefly, CDI patients had clinical diarrhea and tested positive on clinical NAAT performed on their stool, and carriers had recently received antibiotics, did not have diarrhea, and tested positive on NAAT performed on their stool. Samples were also collected under the parent Internal Review Board protocol from pediatric (Boston Children's Hospital) and adult (Texas Medical Center) patients with clinical diarrhea who tested positive for *C. difficile* on routine clinical testing algorithms. Strains were cultured from stool as previously described [13]. Briefly, stool specimens were grown anaerobically on *C. difficile*–selective cycloserine cefoxitin fructose agar (Anaerobe Systems, Morgan Hill, CA), after ethanol shock treatment. Colonies of *C. difficile* were identified on the basis of distinct colony morphology, "horse barn" odor, and Gram stain. For each isolate, in vitro toxin production and activity were assessed by culture and cytotoxicity assay (CTA). The presence of *tcdA* and *B, C. difficile*–specific 16S ribosomal ribonucleic acid and a *C. difficile* housekeeping gene (*tpi*) were further verified by polymerase chain reaction (PCR) [13]. VPI 10463 (A^+B^+), CF2 5340 (A^+B^-) [14] and BAA-1801(A^-B^-) strains were used as controls. The specific primers [15, 16] are shown in Supplementary Table 2.

Preparation of Culture Filtrates

A series of clinical strains previously isolated from cycloserine cefoxitin fructose agar plates were cultured in Difco cooked meat media (Becton, Dickinson and Company, Franklin Lakes, NJ) for 24, 48, and 72 hours. Culture filtrates were prepared by 10 000g centrifugation (10 minutes) to obtain a supernatant, followed by passage through an 0.45 μm syringe filter and then the addition of a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at a ratio of 1:25. Samples were aliquoted and frozen at −80°C before use. The in vitro production of toxins A and B was determined for all isolates by Simoa assays, as described below. The culture filtrate of the high toxin-producing strain VPI 10463 (A^+B^+) was used as a control.

Simoa Assays

The *C. difficile* Simoa assay is an ultrasensitive, quantitative single molecule array immunoassay for the detection and quantification of free toxins A and B in stool samples [12]. Details of the Simoa assays have been described previously [12]. In this study, the Simoa immunoassay was used to measure toxins A and B in clinical stool specimens, culture filtrates, and mouse cecal or colonic aspirate samples. Samples were tested diluted at 1:21 and at 1:1050 for culture filtrate or mouse cecal content samples with toxin A and/or B measurements >100 000 pg/mL at 1:21 dilution. Results are given in pg/ml in the diluted sample (1:21). The limits of detection for toxins A and B in stool (and culture filtrate) were 0.6 and 2.9 pg/mL, respectively.

Animal Model of *Clostridioides difficile* Infection

A mouse model of *C. difficile* infection was utilized as previously described [17]. Briefly, pathogen-free wild-type C57BL/6 mice (female; 6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were housed in groups of 3 per cage under specific, pathogen-free conditions and fed *ad libitum* with standard rodent chow (Purina 5000) and fresh, distilled water. Prior to infection, mice were pretreated with a 5-antibiotic cocktail consisting of kanamycin (40 mg/kg), gentamicin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg), and vancomycin (4.5 mg/kg; Sigma-Aldrich, St. Louis, MO) administered *ad libitum* in drinking water for 3 days. The antibiotic suspension was freshly prepared every day. The concentration of the antimicrobial mixture was calculated based on the average weight of the mice and their expected water consumption. After the treatment, water was switched to regular, autoclaved water for 2 days to avoid residual metronidazole or vancomycin repressing *C. difficile* growth, followed by the intraperitoneal injection of a single dose of clindamycin (10 mg/kg). After a further 24 hours, animals were inoculated intragastrically with 200 μL Difco cooked meat media (BD, Franklin Lakes, NJ), containing 0.5×10^5 colony-forming units of toxinogenic *C. difficile* strain VPI 10463 or study clinical isolates. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee committee of Beth Israel Deaconess Medical Center.

Ribotyping of *Clostridioides difficile* Isolates

Fluorescent ribotyping was performed as previously described [18, 19]. This technique does not distinguish between ribotypes 053 and 163, ribotypes 014 and 020, or ribotypes 078 and 126; therefore, these are reported as combined ribotypes (ie, 053-163, 014-020, and 078-126).

RESULTS

Toxin Detection in Stools of *Clostridioides difficile* Infection Patients and Asymptomatic Carriers

A total of 231 NAAT-positive clinical stool specimens, obtained from 187 CDI patients and 44 carriers at Beth Israel Deaconess Medical Center, were tested for TcdA and TcdB with Simoa

assays, as reported in our prior studies [11, 12]. As shown in Supplementary Table 1 and Supplementary Figure 1, most of the stool samples contained more TcdB than TcdA (the median TcdA:TcdB ratios for the 187 CDI patients and 44 carriers were 0.74 and 0.68, respectively). Unexpectedly, we observed samples that had high concentrations of toxin A but minimal/undetectable toxin B, and further pursued that observation in this study.

Of the 231 patients providing samples, 7 CDI patients (7/187, 3.7%) and 6 carriers (6/44, 13.6%) had stools with detectable concentrations of TcdA (mean 1559.8; range 23–17 422 pg/mL), while TcdB was below the clinical cutoff of 20 pg/mL (mean 7.4; range 2–12.5 pg/mL; Table 1) [11, 12]. The average and median ratios of TcdA:TcdB in these 13 individuals were 177.62 and 17.93 (range 6.3 to 1914.5), indicating that TcdA was produced at substantially higher levels than TcdB. Samples with the same TcdA-predominant pattern were also observed in cohorts of pediatric (Boston Children's Hospital) and adult (Texas Medical Center) CDI patients, with rates of 7.5% and 6.8%, respectively (Supplementary Tables 3 and 4).

Clinical Characteristics of Patients Infected With *Clostridioides difficile* A>>B Variants

The clinical characteristics of 13 Beth Israel Deaconess Medical Center patients infected with the *C. difficile* toxin A>>B variants are shown in Table 1. The mean age was 61.2 years (age range 29–89 years). The majority of the patients (76.9%) were older than 55 years. The mean ages of CDI patients and carriers were 55.7 and 67.5 years, respectively; overall, 9/13 (69.2%) were female. Toxin A:B ratios in carriers were similar to those in CDI cases. Based on the elevated white blood cell count and creatinine measures, as well as a CDI-related intensive care unit admission, patient BID-71 had severe CDI, indicating that an isolate with no detectable toxin B production was associated with severe CDI. The clinical syndromes associated with these A>>B variant strains were similar to what has been generally reported in other clinical studies of CDI. Symptoms ranged from asymptomatic carriage to severe diarrhea.

Molecular Characteristics of *Clostridioides difficile* Isolates

We successfully cultivated 12 *C. difficile* strains from 13 fecal samples (Table 2). (R1A-070 could not be isolated because of an improper sample preservation.) The 12 A^+B^- isolates belonged to 9 different ribotypes (Table 2). The most common ribotype observed was F106 (4 of 12). All 12 isolates carried both *tcdA* and *tcdB* genes. Testing of clinical stool samples by the Xpert assay indicated that neither the binary toxin-encoding gene (*cdtA*) nor an 18-bp deletion at position 117 in the *tcdC* gene were present in any sample.

Detection and Quantification of Toxins in Culture Filtrates of *Clostridioides difficile* Isolates

To examine whether the toxin A>>B phenotype observed in human clinical specimens was also observed in vitro, TcdA and

Table 1. Clinical Summary of Patients Infected or Colonized With *Clostridioides difficile* Toxin Variants (A>>B)

Variable	BID-20	BID-37	BID-71	M-118	M-223	M-391	M-494	M-639	M-687	R1A-007	R1A-070	R1A-086	R1A-122
Sex	F	F	M	M	M	F	F	F	F	F	F	M	F
Age	29	36	58	39	61	63	70	89	83	61	64	68	74
Inpatient vs outpatient	Outpatient	Outpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient	Inpatient
CDI vs carrier	CDI	Unknown[a]	CDI[b]	Carrier	Carrier	Carrier	Carrier	Carrier	Carrier	CDI	CDI	CDI	CDI[c]
C. difficile **toxin concentrations in stool samples**													
TcdA, pg/ml	105.0	115.0	23.0	110.5	61.2	74.5	127.3	358.0	1213.9	53.0	17 422.0	94.7	519.5
TcdB, pg/ml	3.0	4.0	2.0	12.5	6.6	9.1	7.1	76	8.0	8.4	9.1	9.9	8.6
A/B ratio	35.0	28.8	11.5	8.8	9.3	8.2	17.9	47.1	151.7	6.3	1914.5	9.6	60.4
Laboratory findings													
WBC, $\times 10^9$cells/mcL	7.2	11.8	39.9	2.4	15.9	5.3	13.8	8.7	22.2	4.2	6.5	6.8	8.1
Creatinine, mg/dL	Unknown	0.6	2.5	1.2	0.8	1.4	0.8	1.2	0.8	0.6	0.5	1.4	0.9
Albumin, mg/dL	Unknown	Unknown	2.4	3.8	Unknown	Unknown	Unknown	Unknown	2.5	2.9	2.8	3.4	3.3

Frozen stool samples (diluted 1:21) were tested by Simoa for quantitative detection of TcdA and TcdB.

Abbreviations: CDI, *Clostridioides difficile* infection; F, female; ICU, intensive care unit; M, male; WBC, white blood cell.

[a]Stool frequency at the time of sample collection was unknown; thus, CDI status could not be confirmed.

[b]Patient was admitted to the ICU within 40 days of sample collection, and CDI was noted to be a contributing factor to the admission.

[c]Patient was admitted to the ICU within 40 days of sample collection, but CDI was not felt to be a contributing factor.

Table 2. Characterization of *Clostridioides difficile* Isolates

No.	Isolate	*tcdA, tcdB*[a]	Binary Toxin (*cdtA*)[b]	*tcdC* deletion (repressor deletion 117)[b]	Ribotype	Cytotoxicity
1	**BID-20**	+	–	–	F137	+
2	**BID-37**	+	–	–	F106	+
3	**BID-71**	+	–	–	F106	+
4	**M-118**	+	–	–	F056	+
5	**M-223**	+	–	–	F255	+
6	**M-391**	+	–	–	F014-020	+
7	**M-494**	+	–	–	F106	+
8	**M-639**	+	–	–	F014-020	+
9	**M-687**	+	–	–	F002	+
10	**R1A-007**	+	–	–	F106	+
11	**R1A-086**	+	–	–	F001	+
12	**R1A-122**	+	–	–	F053-163	+

We successfully cultured 12 isolates of *Clostridioides difficile* from 13 stool samples (from patients indicated in Table 1).
Abbreviations: -, absence; +, presence.
[a]All strains were analyzed by polymerase chain reaction to verify the presence of *tcdA* and *tcdB* genes.
[b]Analyzed by Cepheid Xpert *Clostridioides difficile/EPI*.

TcdB were measured by Simoa in culture supernatants harvested at 24, 48, and 72 hours of culture. Consistent with the quantification of TcdA and TcdB in clinical stool specimens, the concentrations of TcdA far exceeded those of TcdB in supernatants from in vitro cultures of all 12 strains (A:B median ratio 20.95 and range 8.3 to 930.9 at 24 hours; A:B median ratio 22.5 and range 10.2 to 1678.7 at 48 hours; A:B median ratio 25.7 and range 11.1 to 3577.6 at 72 h; Table 3; Supplementary Figure 3). Under the same culture conditions, the ratios of TcdA:TcdB for *C. difficile* VPI 10463 were 2.3, 1.3, and 1.1 at 24, 48, and 72 hours, respectively. For each isolate, toxin activity in vitro was also assessed by tissue culture CTA; all supernatants tested positive by CTA (Table 2).

Virulence of TcdA>>B Strains in Mouse Model

To further examine these clinical isolates in vivo by a CDI mouse model, we infected groups of healthy C57BL/6 mice (n = 3, per strain) with *C. difficile* VPI 10463 (control) and 8 clinical isolates with variable toxin A:B ratios, based on Simoa toxin measurements in stool samples (BID-20, BID-37, BID-71, M-639, M-687, R1A-122, R1A-086, and R1A-007). Mice infected by VPI 10463 showed typical symptoms of CDI, including weight loss, a hunching posture, and diarrhea, while the clinical manifestations of mice infected with the A>>B variants varied from no evidence of disease to severe or fatal infections (Table 4; Supplementary Figure 4). Colonic tissue segments were examined for pathologic evidence of damage (Supplementary Figure 2). Uninfected control mice did not exhibit any pathological damage (Supplementary Figure 2*A*). On Day 3 postinfection, severe mucosal damage, including edema, infiltration of inflammatory cells, and epithelial cell damage, was evident in VPI 10463 (Supplementary Figure 2*B*) and in 3 of 7 *C. difficile* variant–infected mice examined pathologically (Supplementary Figure 2*C*, R1A-086; Supplementary Figure 2*D*, BID-20; and Supplementary Figure 2*E*, M-687); only minor pathological manifestations were found in mice infected with R1A-122 (Supplementary Figure 2*F*), BID-37 (Supplementary Figure 2*G*), R1A-007 (Supplementary Figure 2*H*), and M-639 (Supplementary Figure 2*I*). The measurement of cecal toxin concentrations in 1 group of variant-infected mice (strain BID-71; n = 3) revealed that cecal toxin concentrations were >200 000 pg/ml toxin A and ~100 pg/ml toxin B.

DISCUSSION

We here report the discovery of clinical pathogenic *C. difficile* strains that produce high levels of toxin A but minimal toxin B. This pattern of toxin production is not rare (>5% of isolates) and has consistently been observed in vitro and in vivo in both humans and mice. Similar to typical A^+B^+ strains, the A>>B clinical isolates were responsible for both clinical infection and colonization in our human study population, and likewise caused a range of clinical phenotypes in mice. Although disease was not recapitulated in the mouse model for all strains that caused disease in humans, we have observed the same phenomenon previously for other unrelated strains [17]. These data suggest that the significance of toxin A in human CDI pathogenesis cannot be overlooked, and have important implications for CDI diagnosis, treatment, and vaccine development.

It is now accepted that both toxins A and B are proinflammatory, cytotoxic, and enterotoxic in the human colon [20]. Their relative importance in the pathogenesis of CDI has been studied extensively but still remains controversial [21]. Earlier animal experiments with purified toxins had indicated that toxin A alone was able to induce *C. difficile* disease pathology, whereas toxin B was believed to act synergistically with toxin A [22]. However, 2 independent studies with genetically

Table 3. Concentrations of TcdA and TcdB (pg/ml) Produced by Isolated *Clostridioides difficile* Strains In Vitro at Different Time Points

Strains	24h	24h	24h	48h	48h	48h	72h	72h	72h
	A, pg/mL	B, pg/mL	A/B ratio	A, pg/mL	B, pg/mL	A/B ratio	A, pg/mL	B, pg/mL	A/B ratio
VPI 10463	>2 000 000.0	887 155.4	**~2.3**	>2 000 000.0	1 503 972.4	**~1.3**	>2 000 000.0	1 763 053.4	**~1.1**
BID-20	5751.8	275.2	**20.9**	27 809.8	1222.9	**22.7**	39 395.2	1737.2	**22.7**
BID-37	1368.4	65.1	**21**	17 028.1	539.8	**31.5**	35 460.6	1095.2	**32.4**
BID-71	1303.2	1.4	**930.9**	10 072.2	6	**1678.7**	33 629.6	9.4	**35776**
M-118	17 278.8	506.3	**34.1**	116 582.8	2944.6	**39.6**	163 083.6	3750.6	**43.5**
M-223[a]	2241.6	148.4	**15.1**	22 710.4	1186.8	**19.1**	30 542.6	1622.8	**18.8**
M-391	2173.2	242.9	**8.9**	15 851.1	1554.2	**10.2**	18 239.2	1646	**11.1**
M-494	1360.4	83	**16.4**	23 760.6	1063.8	**22.3**	29 700.4	1254.4	**23.7**
M-639	3372.3	407.6	**8.3**	19 623.9	1891.6	**10.4**	22 174.6	1949	**11.4**
M-687	10 598.8	465.7	**22.8**	36 865.3	1620.2	**22.8**	48 283.4	1876.8	**25.7**
R1A-007	2906.2	167.2	**17.4**	14 325.6	676.4	**21.2**	18 779.4	713	**26.3**
R1A-086	34 464.2	1000.9	**34.4**	161 605.4	5069.1	**37.7**	181 810.6	5192	**35**
R1A-122[a]	20 190.4	886.6	**22.8**	65 253	3317.4	**19.7**	11 657	410	**28.45**

Strain VPI10463 (a high toxin A and B producer) was used as a control. We incubated 1×10^8 colony-forming units of the vegetative cells of the 12 clinical isolates anaerobically for 24, 48, and 72 hours in cooked meat medium or CCMB-TAL medium. Concentrations of TcdA and TcdB in the resulting conditioned media were determined by Simoa assay.

[a]Incubated in cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme(CCMB-TAL) medium.

Table 4. Diarrhea, Mortality, and Terminal Relative Weight in Mice Infected With Various *Clostridium difficile* Strains

Strains	Diarrhea %, n/N	Mortality %, n/N	Relative Weight %, Death or Day 3
VPI 10463	100 (3/3)	100 (3/3)	87.95
BID-20	100 (3/3)	100 (3/3)	83.17
BID-37	0 (0/3)	0 (0/3)	94.26
BID-71	0 (0/3)	0 (0/3)	105.3
M-639	0 (0/3)	0 (0/3)	96.32
M-687	66.7 (2/3)	33.3 (1/3)	84.89
R1A-122	33.3 (1/3)	0 (0/3)	100.7
R1A-086	100 (3/3)	100 (3/3)	84.23
R1A-007	0 (0/3)	0 (0/3)	97.21

engineered *C. difficile* strains that produced either only toxin A (A^+B^- mutant) or toxin B (A^-B^+ mutant) have yielded conflicting results regarding the importance of toxin A [3, 23]. In 1 study, the authors concluded that toxin B, and not toxin A, is essential for CDI [23]. The second study showed that either mutant was capable of causing disease, but A^-B^+ mutants caused more severe disease [3]. However, the relative importance of these toxins in human CDI cannot be fully predicted from studies using animal models. Our study results challenge the view that toxin B is the sole or dominant virulence factor of *C. difficile*.

Our study has several limitations. First, the main study was conducted at a single center. Although our study population was inclusive of both CDI patients and carriers, these findings needed to be repeated in other cohorts. Notably, we have also observed similar findings (stool samples with toxin A>>B, with toxin B concentrations below our clinical cutoff) in samples from 2 other institutions (Boston Children's Hospital and Texas Medical Center), at similar rates (7.5% and 6.8%, respectively). We also acknowledge that in vitro toxin production does not necessarily reflect toxin production in a human host, but note that we initially discovered these isolates due to our inability to detect toxin B in clinical stool samples using an ultrasensitive assay. We also acknowledge the possibility that toxin B from the A>>B isolates was detected less efficiently (by our Simoa assay reagents) than toxin B from other isolates. However, we preliminarily have not observed any consistent changes in the *tcdB* sequences of these isolates that would support the differential detection of the *tcdB* gene product by our assay reagents [24]. We also note that a separate ultrasensitive assay (using different toxin B detection reagents) also detected a similar phenomenon in 1 clinical sample (high concentration of toxin A but toxin B concentration below their clinical cutoff) [25]. Finally, while we find it notable that the A:B ratios were consistent in both in vivo and in vitro contexts, we acknowledge that the clinical relevance of the toxin A:B ratio is not clear, given that clinical presentation (in humans and in mice) did not obviously correlate with the A:B ratio.

Variation in the *C. difficile* genome is extensive, with new genetic changes being discovered continuously, and the regulation of toxin gene expression is extremely complex [26]. Our ribotyping analysis confirmed that the observed pattern of production of toxin A>>B is possible in a diverse set of isolates, and our data show that these isolates maintain the A>>B phenotype both in vivo and in vitro. Moreover, the frequency of recovery of A>>B isolates in patients with CDI and *C. difficile* colonization in our study indicates that this phenotype is reasonably common, and that it simply has been overlooked previously due to the lack of technologies able to effectively quantify both toxins A and B in clinical and in vitro samples. Our novel data suggest that diagnostic methods that detect the toxin B protein alone may be inadequate to reliably exclude CDI. Our findings also draw into question the prudence of developing therapies that focus solely on neutralizing toxin B. While current molecular tests will detect these A>>B strains, with the advent of ultrasensitive toxin immunoassays, it is important that toxin A detection (and not only toxin B detection) be prioritized.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Q. L., N. R. P., and X. C. contributed equally to this work. N. R. P., C. P. K., and X. C. conceptualized the study and acquired funding. Q. L., A. B., A. L., H. X., L. G., D. N. G., K. W. G., A. J. G.-L., M. Z., L. S., and D. C. D. conducted the laboratory tests and data collection. Q. L., X. C., N. R. P., and C. P. K. wrote the original manuscript. All authors critically commented on the manuscript and approved the final version.

Acknowledgments. The authors thank M. Jahangir Alam, Khurshida Begum, Bradley Endres, and Chris Lancaster at the University of Houston for assistance with the *Clostridioides difficile* ribotyping assay and colleagues Christine Cuddemi, Christine Geer, and Kaitlyn Daugherty for assistance with sample and data collections.

Financial support. This work was supported by the National Institutes of Health/National Institute of Allergy and Infectious Diseases (grant number 1R01AI116596-01 to N. R. P. and C. P. K.), National Institute of Diabetes and Digestive and Kidney Diseases (grant number T32 DK 07760 to C. P. K.), Institut Merieux (funding to N. R. P. and C. P. K.), the Irving W. and Charlotte F. Rabb Award (to X. C.), and the China Scholarship Council (scholarship to Q. L.). Simoa assays were performed by Quanterix and by bioMerieux.

Potential conflicts of interest. D. C. D., M. Z., and L. S. are employees and stock holders of Quanterix Corporation, which sells Simoa kits, instrumentation, and services. C. P. K. has acted as a paid consultant to Artugen, Facile Therapeutics, First Light Biosciences, Finch, Matrivax, Merck, Seres Health, and Vedanta, outside of this study, and has received grant support from Merck. N. R. P. has acted as a paid speaker for Singulex. K. W. G. has received grant support from Merck. X. C. has acted as a paid consultant to Artugen and received grant support from Merck. A. B. and A. L. are employees of bioMérieux. D. N. G. reports personal fees from Merck, Rebiotix, Ferring, Actelion, Summit, DaVolterra, Pfizer, MGB Biopharma, Sanofi Pasteur, and Medpace, all outside the submitted work, and has been issued a patent for Treatment and Prevention of *Clostridioides difficile* Infection. All other authors report no potential conflicts. 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.

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