

Supplementary Materials

High Serum Levels of Toxin A Correlate with Disease Severity in Patients with *Clostridioides difficile* Infection

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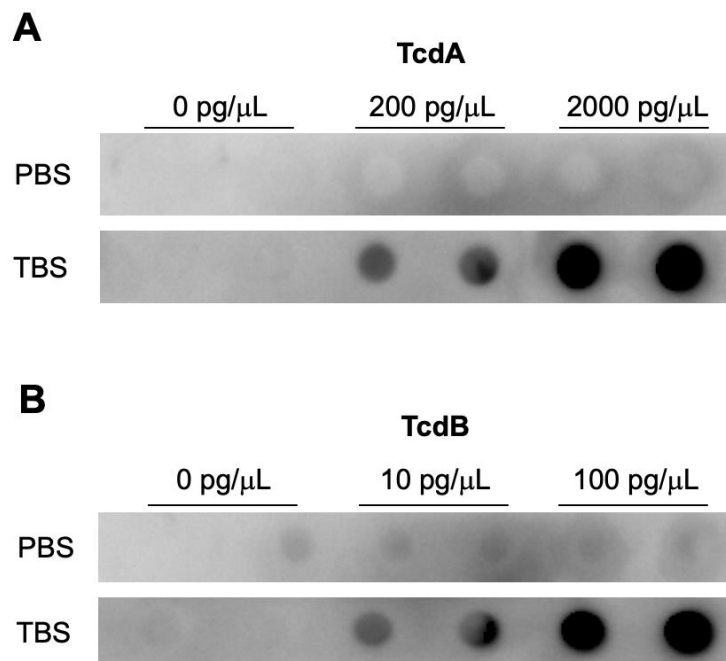


Figure S1. *C. difficile* toxins resuspension in either PBS or TBS is critical for dot-blot sensitivity. Representative images of dot-blot experiment using the commercial (A) TcdA and (B) TcdB toxins resuspended in either PBS or TBS. The resuspension in TBS allowed the solubilization of the serum lipid and consequently a higher sensitivity of the technique. Each toxin concentration was spotted on PVDF membrane in duplicate. Signals were revealed using specific anti-TcdA or anti-TcdB antibodies.

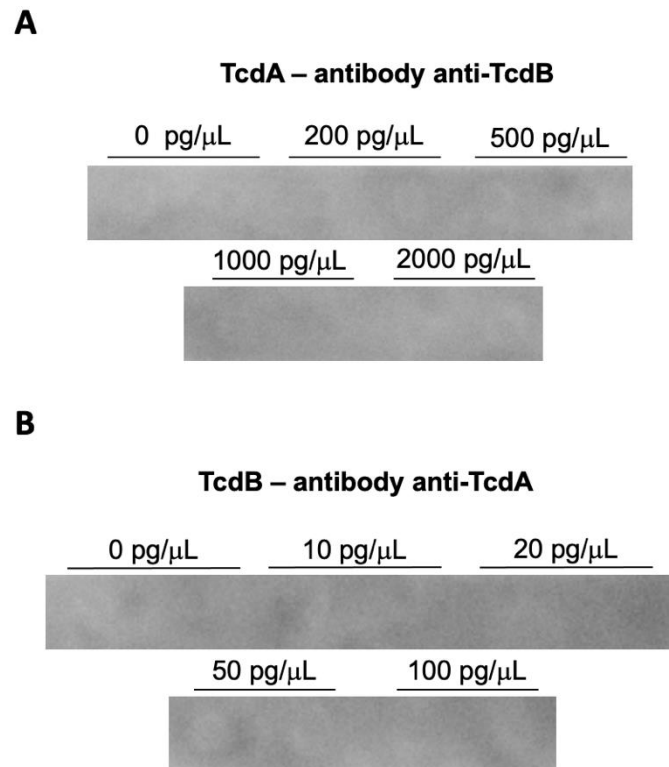


Figure S2. Cross-reactivity experiments to evaluate the specificity of the dot-blot assay. (A) The indicated concentrations of the commercial TcdA were spotted in duplicate and the PVDF filter was blotted using the anti-TcdB specific antibody. (B) The indicated concentrations of the commercial TcdB were spotted in duplicate and the PVDF filter was blotted using the anti-TcdA specific antibody.

Table S1. Demographic and epidemiological data, comorbidities, clinical characteristics, and outcome of the 35 CDI cases included in the study. CCI: Charlson Comorbidity Index; rCDI: recurrent CDI; COPD: chronic obstructive pulmonary disease. SD: standard deviation.

Characteristics	Mean \pm SD (%)
Female gender	18 (51.4%)
Age (years)	60 (range: 19-86)
First CDI episode	30 (85.7%)
Recurrence of CDI	5 (14.3%)
No comorbidities	10 (28.6%)
Comorbidities	25 (71.4%)
Cardiovascular disease	13 (37.1%)
Heart failure	4 (11.4%)
Diabetes	7 (20.0%)
Renal failure	3 (8.6%)
Dialysis	0
Chronic liver failure	2 (5.7%)
Neurological disease	4 (11.4%)
Vasculitis	2 (5.7%)
COPD	9 (25.7%)
Solid cancer	3 (8.6%)
Blood cancer	3 (8.6%)
Transplant, immunodeficiency, immunosuppression	7 (20.0%)
Chronic inflammatory bowel disease	2 (5.7%)
Other concomitant infections	13 (37.1%)
Mean age-adjusted CCI at admission	3.6 (range: 0-8)
Antibiotic in the previous two months	32 (91.4%)
Antiacids in the previous two months	27 (77.1%)
Statins in the previous two months	4 (11.4%)
Steroids in the previous two months	13 (37.1%)
CDI severity	
Mild-moderate	17 (48.6%)
Severe	18 (51.4%)
Patients outcome	
Deceased before the discharge	1 (2.9%)
Recovered at home, no subsequent rCDI	28 (82.4%)
Subsequent rCDI	5 (14.7%)
Deceased, rCDI-related	0
Deceased, no rCDI-related	1 (2.9%)

Table S2. Definitions of CDI, microbiological evidence of CDI, CDI recurrence, mild CDI and severe CDI adopted in the study.

CDI: A clinical picture characterized by diarrhea or ileum or toxic megacolon in the presence of microbiological evidence of CDI.

Microbiological evidence of CDI:

- GDH positive antigen + A/B positive toxins (enzyme immunoassay).
- GDH positive antigen, A/B negative toxins (immunoenzymatic method) + positivity of the amplification of the genes that encode *C. difficile* toxins.
- Positivity of the amplification of the genes that encode *C. difficile* toxins + A/B positive toxins by enzyme immunoassay.

Recurrence of CDI: The onset of a new episode of CDI after at least two days from the resolution of the diarrhea and after the end of the antimicrobial treatment of the previous CDI episode.

Mild CDI: CDI in the absence of the following criteria: fever ($> 38.5^{\circ}$), chills, hemodynamic instability, signs of ileus or peritonitis, leukocytosis (leukocytes $>15,000$ cells/ μ l) creatininemia increase >1.5 times the values before the infection, increase in serum lactates, histological evidence of pseudo-membranous colitis, radiological evidence of ileus or ascites.

Severe CDI: CDI in the presence of at least one of the following criteria: fever ($> 38.5^{\circ}$), chills, hemodynamic instability, signs of ileus or peritonitis, leukocytosis (leukocytes $>15,000$ cells/ μ l) creatininemia increase >1.5 times the values before the infection, increase in serum lactates, histological evidence of pseudo-membranous colitis, radiological evidence of ileus or ascites.

Table S3. TcdA and TcdB plasma levels and presence of the binary toxin *tcdC* gene in the CDI strains found in the 35 patients enrolled in the study. PCR: polymerase chain reaction.

# Patient	T0		T4		T10		Presence of the binary toxin <i>tcdC</i> gene by PCR	Infection Severity
	TcdA (pg/ μ L)	TcdB (pg/ μ L)	TcdA (pg/ μ L)	TcdB (pg/ μ L)	TcdA (pg/ μ L)	TcdB (pg/ μ L)		
001	79,8 \pm 5,8	0,0	24,6 \pm 5	0,0	14 \pm 0,8	0,0	n/a	Mild
002	0,0	51,2 \pm 0,6	0,0	49,6 \pm 1,2	0,0	45,2 \pm 6	n/a	Mild
003	112,4 \pm 14,6	44 \pm 1,6	0,0	22 \pm 0,2	7,4 \pm 1,6	19 \pm 2,2	Negative	Mild
004	214,8 \pm 4	137,4 \pm 1	139,6 \pm 3,2	120 \pm 0,8	0,0	77,4 \pm 4,8	n/a	Severe
005	126 \pm 10	0,0	234 \pm 3,8	0,0	231,4 \pm 68,6	0,0	n/a	Severe
006	258,8 \pm 4,8	0,0	265 \pm 36	0,0	227,8 \pm 1,2	0,0	n/a	Severe
007	243 \pm 7	0,0	239 \pm 1	0,0	217 \pm 30	0,0	Positive	Mild
008	256,8 \pm 53,6	0,0	175,2 \pm 4,8	0,0	192,4 \pm 3	0,0	n/a	Mild
009	300 \pm 82	0,0	178,6 \pm 12,4	0,0	n/a	n/a	n/a	Mild
010	257 \pm 34	0,0	69 \pm 10,4	0,0	17,4 \pm 0,6	0,0	Negative	Mild
011	220 \pm 30	0,0	104 \pm 10	0,0	32,6 \pm 6	0,0	n/a	Mild
012	18,6 \pm 26	0,0	52,8 \pm 38	0,0	54 \pm 18	0,0	Negative	Severe
013	38,4 \pm 20	0,0	30,4 \pm 22	0,0	16,4 \pm 10	0,0	n/a	Mild
014	28,6 \pm 16	0,0	0,0	0,0	0,0	0,0	Negative	Severe
015	25,4 \pm 0,8	0,0	17,6 \pm 12	0,0	0,0	0,0	Positive	Severe
016	70,8 \pm 26	0,0	58,4 \pm 42	0,0	15,6 \pm 0,06	0,0	n/a	Mild
017	117,8 \pm 54	0,0	85,8 \pm 29	0,0	38,8 \pm 30	0,0	n/a	Severe
018	70,8 \pm 2,2	0,0	11,6 \pm 8	0,0	0,0	0,0	Negative	Severe

019	137,2 ± 22	0,0	192,2 ± 4,8	0,0	23,2 ± 16	0,0	n/a	Severe
020	28,8 ± 7	0,0	98,6 ± 24,6	0,0	22 ± 14	0,0	Positive	Severe
021	0,0	0,0	34 ± 2,6	0,0	21,6 ± 7,4	0,0	Positive	Mild
022	0,0	0,0	0,0	0,0	0,0	0,0	n/a	Mild
023	20 ± 14	0,0	57,8 ± 19,2	0,0	28,6 ± 20	0,0	n/a	Severe
024	0,0	0,0	0,0	0,0	0,0	0,0	Positive	Mild
025	n/a	n/a	25,8 ± 0,4	0,0	0,0	0,0	n/a	Mild
026	0,0	4 ± 0,4	55,6 ± 16	12 ± 7,2	84,6 ± 15	90,6 ± 3,8	n/a	Severe
027	0,0	0,0	65,6 ± 30	0,0	58,4 ± 5,2	0,0	n/a	Severe
028	26,8 ± 10,8	0,0	78,4 ± 26	0,0	78 ± 17	0,0	Positive	Severe
029	0,0	0,0	30 ± 2,6	0,0	n/a	n/a	Positive	Mild
030	113,4 ± 5,4	0,0	122,4 ± 11,4	0,0	17,8 ± 12	0,0	Negative	Mild
031	40,2 ± 15,6	0,0	109 ± 12,4	0,0	66,8 ± 12,2	0,0	n/a	Mild
032	10,8 ± 4,4	0,0	38,8 ± 15,2	0,0	33 ± 0,6	0,0	Negative	Severe
033	0,0	0,0	n/a	n/a	n/a	n/a	Positive	Mild
034	196,6 ± 10	0,0	150 ± 22	0,0	n/a	n/a	n/a	Severe
035	124,6 ± 13	0,0	103,8 ± 23,8	0,0	n/a	n/a	n/a	Mild

Table S4. Risk factors for high level of TcdA toxemia (TcdA > 60 pg/ L) at the CDI onset. RR: risk ratio. CI: confidence interval. SD: standard deviation. CCI: Charlson Co-morbidity Index.

	TcdA ≤ 60 pg/ L at T0 (N = 17)	TcdA > 60 pg/ L at T0 (N = 17)	RR (95% CI)	Fisher's test*
Female gender	8 (47.0%)	9 (52.9%)	1.1 (0.5-2.2)	P = 1
Mean age (years)	58.5	59.3	-	P = 0.7
Mean age-adjusted CCI at admission ±SD	3.4 ± 2.5	3.7 ± 3.3	-	P = 0.9
Comorbidities				
No comorbidities	5 (29.4%)	5 (29.4%)	1.0 (0.4-2.1)	P = 1
Cardiovascular disease	5 (29.4%)	8 (47.0%)	1.4 (0.6-3.2)	P = 0.4
Heart failure	0 (0%)	4 (23.5%)	-	P = 0.1
Diabetes	3 (17.6%)	4 (23.5%)	1.2 (0.4-3.0)	P = 1
Renal failure	3 (17.6%)	0 (0%)	-	P = 0.2
Inflammatory bowel disease	2 (11.7%)	0 (0%)	-	P = 0.4
Chronic liver failure	2 (11.7%)	0 (0%)	-	P = 0.4
Neurological disease	2 (11.7%)	2 (11.7%)	1 (0.3-2.8)	P = 1
Vasculitis	1 (5.8%)	1 (5.8%)	1 (0.2-4.1)	P = 1
COPD	6 (35.2 %)	2 (11.7%)	0.5 (0.3-1.0)	P = 0.2
Solid cancer	2 (11.7%)	1 (5.8%)	0.7 (0.3-1.7)	P = 1
Blood cancer	1 (5.8%)	2 (11.7%)	1.5 (0.3-7.9)	P = 1
Transplant, immunodeficiency, immunosuppression	3 (17.6%)	4 (23.5%)	1.2 (0.4-3.0)	P = 1
Other concomitant infections	6 (35.2 %)	7 (41.1%)	1.1 (0.5-2.3)	P = 1
<i>Laboratory findings before CDI diagnosis</i>				
Basal Albumin (g/dL ±SD)	3.3 ± 0.5	3.9 ± 0.6	-	P = 0.02
Basal Creatinine (mg/dL ±SD)	0.7 ± 0.2	0.7 ± 0.2	-	P = 0.4
<i>Laboratory findings at T0</i>				
White blood cell peripheral count (10 ³ cells/μL ±SD)	11.38 ± 5.71	11.38 ± 6.47	-	P = 0.8
Neutrophils peripheral count (10 ³ cells/μL ±SD)	8.02 ± 5.33	8.66 ± 5.71	-	P = 0.7
Creatinine (mg/dL ±SD)	1.0 ± 0.7		-	P = 0.7

Albumin (g/dL \pm SD)	3.2 \pm 0.5	3.5 \pm 0.6	-	P = 0.1
TcdB (ng/mL \pm SD)	0.15 \pm 0.60	0.52 \pm 1.7	-	P = 0.4
Severe CDI	6 (35.2 %)	12 (70.5%)	2.0 (1-4.2)	P= 0.04
Patients outcome				
Deceased	0 (0%)	1 (5.8%)	-	P = 1
rCDI	3 (17.6%)	2 (11.7%)	0.8 (0.3-1.8)	P = 1

Table S5. Risk factors for detectable level of TcdB toxemia (TcdB > 4 pg/μL) at the CDI onset. RR: risk ratio. CI: confidence interval. SD: standard deviation. CCI: Charlson Co-morbidity index.

	TcdB not detectable at T0 (N = 30)	TcdB > 4 pg/mL at T0 (N = 4)	RR (95% CI)	Fisher's test*
Female gender	15 (50.0%)	2 (50.0%)	1.0 (0.7-1.2)	P = 1
Mean age (years)	59.8	52.7	-	P = 0.5
Mean age-adjusted CCI at admission ±SD	3.6 ± 2.9	3.0 ± 3.5	-	P = 0.6
Comorbidities				
No comorbidities	8 (26.6%)	2 (50.0%)	2.4 (0.3-14.7)	P = 0.5
Cardiovascular disease	11 (36.6%)	2 (50.0%)	1.0 (0.8-1.4)	P = 0.6
Heart failure	3 (10.0%)	1 (25.0%)	1.2 (0.6-2.1)	P = 0.4
Diabetes	6 (20.0%)	1 (25.0%)	1.0 (0.7-1.4)	P = 1
Renal failure	3 (10.0%)	0 (0%)	-	P = 1
Inflammatory bowel disease	2 (6.6%)	0 (0%)	-	P = 1
Chronic liver failure	2 (6.6%)	0 (0%)	-	P = 1
Neurological disease	4 (13.3%)	0 (0%)	-	P = 1
Vasculitis	2 (6.6%)	0 (0%)	-	P = 1
COPD	8 (26.6%)	0 (0%)	-	P = 0.5
Solid cancer	3 (10.0%)	0 (0%)	-	P = 1
Blood cancer	3 (10.0%)	0 (0%)	-	P = 1
Transplant, immunodeficiency, immunosuppression	7 (23.3%)	0 (0%)	-	P = 0.5
Other concomitant infections	12 (40.0 %)	1 (25.0%)	0.9 (0.7-1.1)	P = 1
<i>Laboratory findings before CDI diagnosis</i>				
Basal Albumin (g/dL ±SD)	3.6 ± 0.6	3.2 ± 0.3	-	P = 0.3
Basal Creatinine (mg/dL ±SD)	0.7 ± 0.2	0.6 ± 0.1	-	P = 0.2
<i>Laboratory findings at T0</i>				
White blood cell peripheral count (10 ³ cells/μL ±SD)	11.00 ± 5.85	12.65 ± 7.94	-	P = 0.6
Neutrophils peripheral count (10 ³ cells/μL ±SD)	8.11 ± 5.29	10.12 ± 7.17	-	P = 0.4
Creatinine (mg/dL ±SD)	1.0 ± 0.6	0.8 ± 0.4	-	P = 0.4
Albumin (g/dL ±SD)	3.4 ± 0.5	3.2 ± 0.6	-	P = 0.5
TcdA (ng/mL ±SD)	4.68 ± 4.84	4.09 ± 5.16	-	P = 0.8

Severe CDI	16 (53.3%)	2 (50.0%)	0.9 (0.7-1.2)	P= 1
Patients outcome				
Deceased	1 (3.3%)	0 (0%)	-	P = 1
rCDI	4 (13.3%)	1 (25.0%)	1.1 (0.7-1.7)	P = 0.4
