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Development of a new serological assay for the diagnosis of *Clostridium difficile* infections with prognostic value

Felix von Bechtolsheim[e], Adorjan Varga[a,c], Laszlo Szereday[a,b], Beata Polgar[a,b], Timea Balassa[d], Bela Kocsis[a], Zoltan Peterfi[c], Eva Miko[a,b,*]

[a] Department of Medical Microbiology and Immunology, Medical School, University of Pecs, Pecs, Hungary
[b] Janos Szentagothai Research Centre, Pecs, Hungary
[c] 1st Department of Medicine, Division of infectious diseases, Medical School, University of Pecs, Pecs, Hungary
[d] Department of Medical Biology and Central Electron Microscope Laboratory, Medical School, University of Pecs, Pecs, Hungary
[e] Department of Visceral-, Thoracic- and Vascular Surgery, University Hospital, Technical University Dresden, Germany

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ABSTRACT

Purpose: The most common hospital-acquired enteral infection is caused by *Clostridium difficile*. Unfortunately, *Clostridium difficile* infections (CDI) are of high risk to recur and little is known about how to predict recurrences. Previous findings have shown that high risk for recurrence correlates with low levels of *C. difficile* toxin-A and -B specific antibodies suggesting the protective role of humoral immunity against bacterial virulence factors. Therefore, the aim of this study was to develop an immunoassay, which specifically measures *C.difficile* toxin-specific antibodies in the serum that might be correlated with the risk of recurrence.

Methods: We developed a simple ELISA to measure the quantity of toxin-A and -B-specific antibodies in human serum. The assay was then used to test anti-toxin immune response in healthy controls, in patients with primary CDI and patients with CDI recurrence.

Results: The developed assay is simple, reproducible and fast. When using this test in a small clinical trial our results showed a trend toward a higher antibody level in those patients with only one episode of CDI, whereas patients with recurrent CDI had less anti-toxin A or B-specific antibodies in their serum indicating inadequate *C. difficile* anti-toxin immunity may facilitate recurrent infections.

Conclusions: It has already been observed that low antibody levels are associated with recurrent CDI (Bauer et al., 2014). The findings of our clinical trial show a similar trend. Our developed ELISA test could help to conduct further research and it might be helpful in clinical use to detect patients of high risk for CDI recurrence.

1. Introduction

Clostridium difficile is the most common cause of hospital acquired diarrhea, a major health concern and financial burden worldwide (Davies et al., 2014; Kwon et al., 2015; Martin et al., 2016). Even though it was discovered and described already in 1935 for the first time, it took more than 40 years to link this pathogen to its various effects on human health. The hypervirulent strain B1/NAP1/027 of this Gram-positive anaerobic spore-forming bacterium is primarily responsible for most of the recent outbreaks (Curry et al., 2007; Loo et al., 2005; McEllistrem et al., 2005). The highly transmissible and resistant spore enables the bacterial entry into the hospital environment and its continuous presence may lead to colonisation of hospitalized patients. The clinical manifestation of the infection has a wide spectrum with high mortality rates. Particularly worrying is the fact, that in approximately 20–30% of patients with primary infection symptoms recur and recurrence rates are even higher after secondary and tertiary *C. difficile* infections (CDI) (Deshpande et al., 2015).

Although the pathogenesis is multifactorial, the cytopathogenic effect of the two exotoxins, A and B is thought to be one of the major virulence factors of *C.difficile* along with being able to produce an endospore (Monaghan, 2015).

Considering that the risk of recurrence is up to 65% in certain patient populations it would be very useful to pre-estimate this risk and adapt the therapy to the given condition. Therefore, the study aimed to develop an immunoassay, which specifically measures *C.difficile* toxin-specific antibodies in the serum that might be correlated with the risk of recurrence. The protocol should be reliable in sensitivity and

* Corresponding author at: Department of Medical Microbiology and Immunology, Medical School, University of Pecs, Pecs, Hungary.
E-mail address: miko.eva@pte.hu (E. Miko).

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Table 1
Testing of different coating buffers. OD values of coated and non-coated control wells using different coating buffers and signal-to-noise values (O.D. of coated positive – O.D. of non-coated control wells) are indicated.

	TRIS buffer (pH 6,5)	Carbonate-buffer (pH 9,6)	PBS buffer (pH 7,4)
Toxin-A	Coated positive: 2694	Coated positive: 2524	Coated positive: 2908
	Coated negative: 0,053	Coated negative: 0,001	Coated negative: 0,005
	Non-coated control: 0,604	Non-coated control: 0,826	Non-coated control: 0,830
	Signal to noise: **2090**	Signal to noise: 1698	Signal to noise: 2078
Toxin-B	Coated positive: 3942	Coated positive: 3562	Coated positive: 3810
	Coated negative: 0,000	Coated negative: 0,005	Coated negative: 0,006
	Non-coated control: 0,113	Non-coated control: 0,164	Non-coated control: 0,161
	Signal to noise: **3829**	Signal to noise: 3398	Signal to noise: 3649

specificity, fast, uncomplicated and if possible, inexpensive. Furthermore, the developed ELISA-assay will be tested in a small clinical trial to assess the benefits and disadvantages of the test itself.

2. Materials and methods donors and patients

Serum samples were collected by clinicians at the 1st Department of Medicine, Division of infectious diseases, Medical School, University of Pécs from December 2014 to May 2016. For measuring physiological *C. difficile* antibody levels, 15 serum samples were collected from anonymous blood donors without any history of *C. difficile* infection based on a detailed questionnaire. Additionally, 47 sera of patients with the diagnosis of CDI were analysed (age range 48–90; 29 women and 18 males). The diagnosis of CDI was based on the symptom diarrhea and *C. difficile* toxin detection in the stool with an immune chromatography test. Blood samples were drawn on the 8th day of antibiotic therapy. After discharge, patients were followed up in order to notice recurrent CDI. According to a subsequent CDI recurrence (more than two weeks and less than eight weeks following the onset of the primary episode), patients were grouped in “primary CDI” and “recurrent CDI” groups. Serum samples were stored at −80 °C until carrying out the experiments. Informed consent was taken from each donor and patient. The study was approved by the Local Ethical Committee of the Medical School of the University of Pecs.

3. Materials

For the development of a *C. difficile* Toxin A and B antibody capture-ELISA test, the following reagents were used: for microplate: NUNC-MaxiSorp Immunoplate F96 (Thermo Scientific); for buffers: Trizma base (TRIS, Sigma Aldrich), Dulbecco's PBS without Ca and Mg (PAA Laboratories GmbH); for coating: *C. difficile* Toxin A and Toxin B (Enzo Life Sciences), for blocking: bovine serum albumin (BSA, Sigma Aldrich), Superblock (PBS) Blocking Buffer (Thermo Scientific Inf.). Antibodies used in the ELISA test: primary antibody: rabbit polyclonal antibody to *C. difficile* Toxin A and Toxin B (Abcam), secondary antibody: polyclonal goat anti-rabbit immunoglobulin-HRP (DAKO), polyclonal goat anti-human immunoglobulin-HRP (DAKO). For detection: BD OptEIA TMB Substrate Reagent Set (Beckton Dickinson).

4. ELISA development

4.1. Testing of different coating buffers for C. difficile anti-toxin capture ELISA

For initial coating concentration 1 μg/ml protein was chosen for both *C. difficile* toxins. Three coating buffers with different pH were tested: TRIS buffer (pH 6,5), carbonate buffer (pH 9,6) and PBS buffer (pH 7,4). Immunoplates were coated with 100 μl/well of Toxin A and Toxin B diluted in the different coating buffers and incubated overnight at 4 °C. After 3 x washing with PBS supplemented with 0,05% Tween washing buffer, wells were blocked with 200 μl/well of PBS supplemented with 0,5% gelatine and 0,1% BSA for 1 h at 37 °C. Then plates were washed with 3 × 200 μl of PBS-Tween washing buffer and rabbit polyclonal antibody to *C. difficile* Toxin A and Toxin B (at a dilution of 1: 100) and polyclonal goat anti-rabbit immunoglobulin (Ig)-HRP (at a dilution of 1: 2000) were applied as primary and secondary antibodies. The plates were incubated for one hour at 37 °C. Next, the wells were washed again to remove the unbound antibody-enzyme conjugates and TMB chromogen/substrate was added to develop the reaction. After 30 min (Toxin A) or 10 min (Toxin B) incubation at room temperature (RT) in the dark, the color development was stopped by adding 100 μl of 1 M H_2SO_4 to the wells. The optical density (O.D.) of the coated positive (toxin coated wells +1:100 rabbit polyclonal antibody to Toxin A/B + anti-rabbit Ig-HRP), coated negative (toxin coated wells + anti-rabbit Ig-HRP) and non-coated control wells (non-coated wells +1:100 rabbit polyclonal antibody to Toxin A/B + anti-rabbit Ig-HRP) were determined at 450 nm with BMG Optima spectrophotometer.

As shown in Table 1., TRIS-buffer provoked the lowest nonspecific reaction in the non-coated wells and gave the highest specific signal to noise value (O.D. of coated positive – O.D. of non-coated control wells) in both Toxin A and Toxin B ELISA.

4.2. Choosing the blocking buffer

In order to test the accuracy of the immunoassay, a collection of blood samples of definitely non-infected (negative) and definitely infected (positive) patients were gathered. The collected samples were pre-tested with *C. difficile* anti-toxin specific ELISA, then a negative and a positive serum pool were created by mixing the three most positive and three most negative samples, respectively. The obtained serum pools were used in further setup assays.

For setup of blocking conditions three different blocking solutions were tested: skimmed milk solution (3%), BSA (5%) and Superblock buffer (Thermo Scientific). ELISA-plates were coated with 1 μg/ml Toxin A and 0,5 μg/ml Toxin B in TRIS-buffer (pH 6,5) overnight at 4 °C. The next day the immunoplates were washed three times with PBS-Tween and 200 μl per well of skimmed milk and BSA solution were added to the wells for 1 h at 37 °C. According to the manufacturer's instruction, the Superblock buffer remained for 30 min on the ELISA-plates to block non-specific binding sites. After washing, 1:100 diluted positive and negative serum pools were applied to the toxin-coated and non-coated wells and incubated for 1 h at 37 °C. Then the plates were washed again and goat anti-human Ig-HRP (1:30000) was added to the wells. After one hour incubation at 37 °C, the reaction was developed by TMB.

As indicated in Table 2, the choice was made in favour of Superblock solution since this buffer resulted in the highest signal to noise value in the case of Toxin B (O.D. of toxin coated No1 wells – O.D. of non-coated No1 wells). Although 5% BSA solution was also effective, skimmed milk solution (3%) showed less satisfying results in the experiments.

Table 2
Testing of different blocking conditions. OD values of coated and non-coated wells as well as the signal-to-noise values (O.D. of coated positive – O.D. of non-coated control wells) are indicated.

	Toxin coated wells			Non-coated wells			Signal to noise
	1	2	3	1	2	3	
Toxin-A							
Skimmed milk (3%)	0,555	0,012	0,011	0,348	0,007	0,010	**0,207**
BSA (5%)	0,416	0,032	0,002	0,239	0,002	0,000	0,177
Superblock	0,573	0,021	0,002	0,376	0,004	0,000	0,197
Toxin-B							
Skimmed milk (3%)	1464	0,007	0,005	0,361	0,000	0,001	1103
BSA (5%)	1331	0,012	0,002	0,258	0,042	0,005	1077
Superblock	1933	0,075	0,070	0,522	0,066	0,069	**1411**

Control wells:
1: + 1:100 positive serum pool +1:30000 goat anti-human Ig-HRP.
2: + 1:30000 goat anti-human Ig-HRP.
3: + 1:100 positive serum pool.

4.3. Cross-titration

The concentration of each component of the immunoassay was optimized by chessboard titration (CBT). During this process the amount of coating antigen and the dilution of primary antibody were tested against each other on a microtiter plate to examine the activities inherent at all the resulting combinations. In the case of this assay, three main test components - the antigen (*C. difficile* Toxin A/B), the primary antibody (positive and negative serum pool) and anti-species conjugate (goat anti-human Ig-HRP) - needed to be optimized.

1. Step: Titration of coating antigen to positive and negative test sera, using a goat anti-human Ig-HRP conjugate at a dilution recommended by the manufacturer.

First Toxin A (titration range: 2–0,125 µg/ml) and Toxin B (titration range: 1–0,0625 µg/ml) were diluted in TRIS buffer (pH 6,5) and test plates were coated with 100 µl/well of diluted antigens from column No1 to 5 for positive sera, and column No7 to 11 for negative sera. Column No6 and 12 received coating buffer only. After overnight incubation at 4 °C, plates were washed three times with 200 µl PBS-Tween washing buffer (pH 7,4) and blocked with 200 µl/well of Superblock reagent for 30 min at room temperature to inhibit non-specific absorption of proteins. Then ELISA plates were washed again and two-fold serial dilutions of pooled positive and negative sera (titration range: from 1:100 to 1:3200 diluted in washing buffer) were added to the plate rows from B to G. Row A and H received sample diluent only. After incubation for 1 h at 37 °C, plates were washed three times with washing buffer. Next 100 µl/well of goat anti-human Ig-HRP conjugate was added to the sample wells at a single dilution of 1:30000 in PBS-Tween supplemented with 0,05% BSA and incubated subsequently for one hour at 37 °C. Following three time washing, 100 µl/well of TBM chromogen/substrate was pipetted into each well and incubated for 30 min at 23 °C in the dark. The color reaction was stopped by adding 100 µl/well of 1 M H_2SO_4 to each well and optical density was determined at 450 nm by a BMG Optima spectrophotometer. The differences between O.D. values of positive and negative sera as well as the binding ratios (O.D. values of pooled positive sera/ O.D. values of pooled negative sera) were determined.

In anti-Toxin-A CBT, the best binding ratio (3921) was observed when test wells were coated with 2 µg/ml of Toxin A and the pooled positive and negative sera were used at a dilution of 1:100 (Table 3). In the case of Toxin-B CBT assay, the highest binding ratio was seen when 1 µg/ml (18,230) or 0,5 µg/ml (18,036) of Toxin B protein was used for coating and 1:800 serum dilution was applied during the assay

Table 3
Immunoreactivity of pooled positive and negative serum samples with *C. difficile* Toxin A antigen. Table 3a: Measured O.D. values of positive serum pool on *C. difficile* Toxin A coated immunoplate. 3b: O.D. values of negative serum pool. 3c: Calculated binding ratios (O.D of positive serum pools/O.D of negative serum pools). The concentration of antigen used for titration is indicated on the top line of the table.

µg/ml	2	1	0,5	0,25	0,125	- Coating
3a. POSITIVE SERUM POOL						
Blank	0,080	0,068	0,074	0,080	0,077	0,073
1:100	0,745	0,558	0,413	0,322	0,259	0,211
1:200	0,442	0,324	0,282	0,192	0,163	0,139
1:400	0,278	0,213	0,213	0,126	0,108	0,095
1:800	0,175	0,123	0,134	0,070	0,060	0,064
1:1600	0,115	0,080	0,089	0,046	0,056	0,045
1:3200	0,088	0,065	0,070	0,037	0,033	0,047
3b. NEGATIVE SERUM POOL						
Blank	0,070	0,086	0,082	0,083	0,075	0,074
1:100	0,190	0,167	0,214	0,215	0,163	0,193
1:200	0,130	0,112	0,164	0,098	0,109	0,142
1:400	0,090	0,077	0,100	0,077	0,085	0,111
1:800	0,070	0,051	0,067	0,042	0,067	0,088
1:1600	0,050	0,042	0,050	0,045	0,055	0,070
1:3200	0,050	0,039	0,057	0,039	0,039	0,070
3c. BINDING RATIOS						
Blank	1143	0,791	0,902	0,964	1027	0,986
1:100	**3921**	3341	1930	1498	1589	1093
1:200	3400	2893	1720	1959	1495	0,979
1:400	3089	2766	2130	1636	1271	0,856
1:800	2500	2412	2000	1667	0,896	0,727
1:1600	2300	1905	1780	1022	1018	0,643
1:3200	1760	1667	1228	0,949	0,846	0,671

Table 4
Immunoreactivity of pooled positive and negative sera with *C. difficile* Toxin B. Table 4a: Measured O.D. values of positive serum pool. 4b: O.D. values of negative serum pool. 4c: Calculated binding ratios (O.D of positive serum pools/ O.D of negative serum pools). The concentration of antigen used for titration is indicated on the top line of the tables.

µg/ml	1	0,5	0,25	0,125	0,0625	- Coating
4a. POSITIVE SERUM POOL						
Blank	0,076	0,075	0,071	0,067	0,079	0,077
1:100	4425	3337	2744	2016	1466	0,877
1:200	3558	2867	2232	1565	1049	0,565
1:400	2824	2216	1674	1148	0,745	0,345
1:800	2060	1497	1066	0,728	0,475	0,187
1:1600	1318	0,869	0,606	0,446	0,289	0,096
1:3200	0,760	0,501	0,351	0,252	0,159	0,047
4b. NEGATIVE SERUM POOL						
Blank	0,075	0,085	0,076	0,079	0,077	0,069
1:100	0,391	0,342	0,319	0,295	0,294	0,298
1:200	0,263	0,209	0,295	0,182	0,179	0,196
1:400	0,187	0,144	0,134	0,112	0,116	0,116
1:800	0,113	0,083	0,084	0,061	0,063	0,090
1:1600	0,092	0,056	0,060	0,048	0,051	0,066
1:3200	0,079	0,037	0,052	0,023	0,035	0,058
4c. BINDING RATIOS						
Blank	1013	0,882	0,934	0,848	1026	1116
1:100	11,317	9757	8602	6834	4986	2943
1:200	13,529	13,718	7566	8599	5860	2883
1:400	15,102	15,389	12,493	10,250	6422	2974
1:800	**18,230**	**18,036**	12,690	11,934	7540	2078
1:1600	14,326	15,518	10,100	9292	5667	1455
1:3200	9620	13,541	6750	10,957	4543	0,810

(Table 4). Finally, the coating concentration of 0,5 µg/ml of Toxin B antigen was chosen making it possible to distinguish accurately between specific immunoreactivity of positive serum and background binding of the negative serum samples.

2. Step: Titration of secondary antibody to a single dilution of pooled

Table 5
Determination of optimal secondary antibodies dilution used for *C. difficile* Toxin A and B specific antibody capture immunoassay. O.D. values of pooled positive serum samples on Toxin A and B antigen coated plate and signal-to-noise values (O.D. of coated positive – O.D. of non-coated wells) are indicated.

Secondary antibodies	Toxin A			Toxin B		
	1:30000	1:40000	1:50000	1:30000	1:40000	1:50000
Coated wells	2108	1626	1307	3558	3160	2814
Non-coated wells	1485	1120	0,891	0,980	0,740	0,577
Signal to noise	**0,623**	0,506	0,416	**2578**	2420	2237

positive sera on a Toxin A and B coated immunoplate.

The test conditions of the assays were chosen during the initial CBT and were as follows: coating buffer: TRIS (pH 6,5); antigen: 1 µg/ml for Toxin A and 0,5 µg/ml for Toxin B; coating: overnight at 4 °C; blocking buffer: Superblock buffer for 30 min at room temperature. Pooled positive serum was used at a dilution of 1:100 for Toxin-A and 1:800 for anti-Toxin B ELISA. The secondary antibody (goat anti-human Ig-HRP conjugate) was tested at a dilution of 1:30000,1:40000 and 1:50000. Since the 1: 30000 dilution gave the highest specific signal-to-noise value we chose this concentration for the final assay (Table 5).

Final ELISA-Protocol:

Coating antigen to microplate: Therefore 2 µg/ml Toxin-A and 0,5 µg/ml Toxin-B are separately diluted in TRIS-buffer (pH 6,5). The ELISA-microplate is coated and incubated overnight at 4 °C.

Washing: The coating liquid was removed and all wells were washed. Therefore 200 µl PBS supplemented with 0,05% Tween was filled in each well, then the solution was removed by flicking the plate over a sink. Remaining drops were removed by patting the microplate upside down on a paper towel. This whole procedure was repeated three times.

Blocking: 200 µl/well Superblock blocking solution was added and incubated for 30 min at room temperature. Then the microplate was washed again with PBS-Tween three times.

Incubation of primary antibody: The serum of the patients was diluted 1:100 for Toxin A and 1:800 for Toxin B in PBS-Tween (PBST). Then 100 µl of the solutions were filled in the adequate wells and empty wells were filled only with PBST buffer. The microplate was incubated for 60 min at 37 °C. In the meantime the PBST-BSA solution for the secondary antibodies was prepared by dissolving 0,2 g BSA in 40 ml PBS-Tween. The PBST-BSA solution was incubated in 37 °C until the primary antibody was ready for continuing the protocol.

Incubation of secondary antibody: The washing procedure was performed three times before continuing with preparing the 1:30000 goat anti-human Ig-HRP secondary antibody solution in PBST-BSA. 100 µl of the diluted secondary antibody solution was filled in the adequate wells, the empty wells were filled with the PBS-BSA-solution without antibody. The microplate was then incubated again for 60 min at 37 °C.

Visualisation: The liquid was removed and the wells were filled with 200 µl PBS supplemented with 0,05% Tween for washing. This step was repeated two more times. Each well was filled with 100 µl of TMB chromogen/substrate solution. Then the microplate was covered against light and incubated for 30 min at 23 °C.

Stoping the reaction: The reaction was stopped by adding 100 µl of 1 M H_2SO_4 in each well.

Measuring: The light-absorption was measured at 450 nm by BMG Optima spectrophotometer.

Statistical analysis of the data: In order to compare data of all groups, multiple analysis with software SPSS version 20 was performed. Multiple comparisons were made using the one-way ANOVA with Bonferroni correction. Differences were considered significant if the *P*-value was equal to or less than 0,05.

5. Results

The development of an ELISA which can specifically test for antibodies only against toxigenic *C. difficile* strains was successful. Moreover, our test was able to determine the level of toxin-specific antibody in patients serum and could distinguish between toxin-A and toxin-B specific immunoreactivity. The major advantages of our method are its short turnaround time and its easy handling. Furthermore, since serum samples are easy to store, therefore later repeating the test or re-evaluation of the obtained result is also possible. On the other hand, the average cost of the assay /patient - is not yet calculable, since this ELISA is not an industrial testing kit.

For the development of the ELISA assay we examined many different materials and reagents. We also tested various materials and concentrations for coating buffers, blocking solutions and toxin-dilution (Table 6). All of these trials were counterchecked by using positive and negative serum controls. Small adjustments to the incubation time were also made.

In the next step, 62 serum samples were tested. The age of all patients was 66,4 years in average. The control group with 43,5 (22–58) years was significantly younger than the primary CDI and recurrent CDI group with 72,4 (34–90) and 75,7 (64–88) years, respectively.

ELISA assay measures optical density, which in the case of antibody-capture ELISA correlates with the amount of antibodies in the serum. Therefore, it is possible to make a statement about the quantity of antibody present in the serum. In our first test series, the *p*-value was above 0,05 and thus obtained results were not significant. Nevertheless, we can report some interesting trend which can be seen in the obtained results as follows: (1) Primary CDI patients seem to have a considerably higher level of antibodies against *C. difficile* toxin-A and toxin-B antigens, compared to the non-infected healthy control and recurrent CDI group (Fig. 1, Table 7). (2) Interestingly, levels of toxin-B specific antibodies did not deviate as much in the control and recurrent CDI group, but varied widely in the primary CDI group. (Table 8). While in the control and recurrent CDI groups toxin B-specific antibody levels were quite low, the primary CDI group outmatched their levels by far in average (Fig. 2).

6. Discussion

Using ELISA techniques for measuring serum antibody responses quantitatively is a widely accepted and traditional method both in diagnostic as well as in research areas. In the case of *C. difficile* immunity, a commercial serological test is not available up to this point. Several independent developed and standardized non-commercial ELISA tests

Table 6
Tested materials during assay development.

	Test components		
	Puffer	Blocking solutions (concentration)	Toxin-dilutions in TRIS-buffer: toxin (µg/ml) + patients serum
Tested materials	TRIS-buffer (pH 6,5) Carbonate-puffer (pH 9,6) PBS-puffer (pH 7,4)	Gelatine (0,5%) BSA (0,1- 1 – 3 - 5%) Skimmed milk (0,1- 1 – 3 - 5%) Superblock	Toxin-A (0,125 – 0,25 – 0,5 – 1 - 2 µg/ml) Toxin-B in TRIS-buffer (0,625–0,125–0,25–0,5–1 µg/ml)

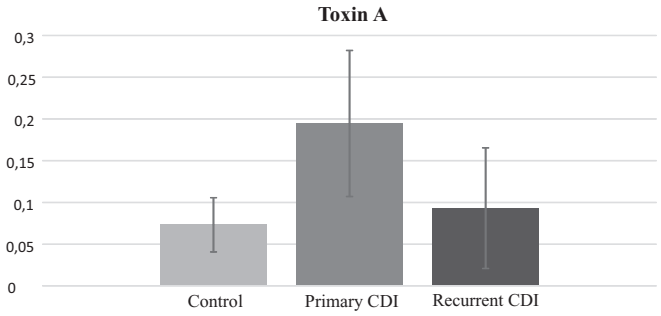

Fig. 1. Graphic presentation of the amount of antibodies against Toxin-A. y-axis: Optical density by 450 nm.

Table 7
Statistical evaluation of antibody quantity against Toxin-A per group.

Toxin-A	Control Group	Primary CDI	Recurrent CDI
Mean	0,07	0,19	0,09
n (persons)	15	26	20
STDEV	0,13	0,45	0,32
SEM	0,03	0,08	0,07

STDEV: standard deviation, SEM: standard error of the mean.

Table 8
Statistical evaluation of antibody quantity against toxin-B per group.

Toxin-B	Control group	Primary CDI	Recurrent CDI
Mean	0,01	0,18	0,03
n (persons)	15	27	19
STDEV	0,02	0,43	0,03
SEM	0,004	0,08	0,01

STDEV: standard deviation, SEM: standard error of the mean.

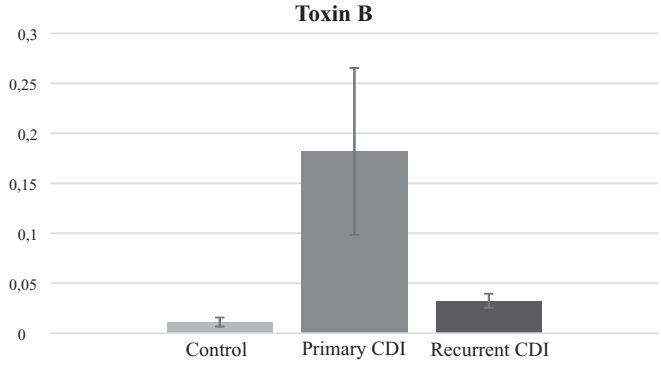

Fig. 2. Graphic presentation of amount of antibodies against toxin-A. y-axis: Optical density by 450 nm.

exist for research purposes and deliver reliable data about host systemic immune response following *C.difficile* infection (Bauer et al., 2014; Johnson et al., 1992; Kyne et al., 2001; Negm et al., 2017; Sanchez-Hurtado et al., 2008). However, current knowledge on this topic is very limited and sometimes even controversial. For a better understanding and future research of humoral immunity against *C.difficile*, we aimed at developing a traditional standardized sandwich ELISA method for detecting human serum *C.difficile* toxin-A and -B specific antibodies.

As a result of our work, we successfully established a useful ELISA protocol to detect and measure *C. difficile* Toxin-A and Toxin-B specific antibodies in human serum. The estimated turnaround time is 6–7 h, which is acceptable for clinical use. As mentioned earlier, it is of advantage that the test detects only toxin specific antibodies. Only a couple of studies have investigated the immune response against non-toxin antigens, e.g. surface layer proteins demonstrating comparable antibody levels in CDI patients, asymptomatic carriers and healthy controls (Bauer et al., 2014; Drudy et al., 2004; Kelly and Kyne, 2011; Sanchez-Hurtado et al., 2008; Wright et al., 2008).

As a result of intestinal colonisation and/or repeated exposure to the environmental bacterium, even healthy individuals may carry *C. difficile* specific antibodies (Salcedo et al., 1997; Viscidi et al., 1983). However, antibody levels will further rise during and after *C. difficile* infection. Several clinical studies confirmed the protective role of adequate humoral immunity in the course of CDI and possible recurrence. For this reason, intravenous immunoglobulin has been used off-label for CDI treatment (Abougergi and Kwon, 2011; Negm et al., 2017; O'Horo and Safdar, 2009; Salcedo et al., 1997; Shah et al., 2014). It was demonstrated that serum anti-toxin A IgG response to the previous colonisation with *C. difficile* may reduce the risk of developing symptomatic CDI in the future (Kyne et al., 2000). Furthermore, during primary CDI, higher levels of toxin A specific IgM and IgG were found to be protective against recurrent infection and median IgG titer was associated with 30 day all-cause mortality (Kyne et al., 2001; Solomon et al., 2013). Other studies revealed the protective role of high serum toxin B antibody concentration as well (Bauer et al., 2014; Gupta et al., 2016; Leav et al., 2010). In cystic fibrosis patients, high anti-toxin antibody levels may explain the rare occurrence of symptomatic *C.difficile* infections among this vulnerable population (Monaghan et al., 2013, 2017).

In our hands, both anti-toxin A and anti-toxin B antibody levels were detectable in the sera of healthy blood donors. Compared to these controls, patients with primary CDI showed elevated levels of toxin A and toxin B specific antibodies but the difference did not reach the level of significance. In patients with recurrent CDI, antibody levels decrease to the level of healthy controls in both cases of toxin A and toxin B. Our results are in line with previous findings discussed above, where similar, but significant changes of humoral immunity against *C. difficile* regarding primary infection and recurrence were described. Limitations of the study are the small sample size of the groups and age difference between the control group and CDI patients, since CDI patients were significantly older than healthy blood donors. However, current knowledge suggests no influence of age on serum antibody levels (Kyne et al., 2000, 2001).

Considering our findings and final assumption of a correlation between decreased antibody levels and recurrence of CDI, our developed ELISA test could help to conduct further research and it might be helpful in clinical use to detect patients of high risk for CDI recurrence.

Ethical approval

All procedures performed were in accordance and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and ethical approval was obtained from the local ethical committee of the Medical School of the University of Pécs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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