Original Article

Rapid and fully-automated detection of *Clostridium difficile* Toxin B via magnetic-particle-based chemiluminescent immunoassay

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Abstract: *Clostridium difficile* colitis is caused by a cytotoxin produced by the anaerobic bacteria *C. difficile* on the epithelial cells of the large intestine, particularly *C. difficile* toxin B (Tcd B). Current *C. difficile* toxin assays have proven to be insensitive and have thus been ruled out from diagnostic purposes. Therefore, Tcd B detection via sandwich-type chemiluminescent immunoassay was proposed as a straightforward approach with potential diagnostic applicability. Here, two high-affinity anti-Tcd B monoclonal antibodies were successfully identified and implemented in a fully-automated magnetic-particle-based chemiluminescent immunoassay (CLEIA). In this test, toxin B was sandwiched between the anti-toxin B antibody-coated magnetic particles and alkaline phosphate-labeled anti-toxin B antibodies. Compared with traditional techniques, the proposed immunoassay demonstrated high sensitivity for toxin B identification and was further optimized to achieve a linear response ranging from 0.12 to 150 ng/mL with a limit of detection (LOD) of 0.47 ng/mL. Importantly, the entire process could be completed in less than 30 minutes. The proposed assay was used to detect toxin B in 104 randomly-selected human stool samples and delivered similar results to those of a commercial ELISA kit, highlighting its great potential for rapid and efficient toxin B determination in human stool specimens.

Keywords: Toxin B, high-affinity antibodies, automation, chemiluminescent immunoassay, point-of-care tests, clinical application

Introduction

*Clostridium difficile* (*C. difficile*), a strictly anaerobic, spore-forming, Gram-positive bacillus, is considered to be the main pathogen responsible for the widespread occurrence of antibiotic-associated diarrhea in hospitals. *C. difficile* infection (CDI) is the primary cause of diarrhea in medical facilities, often affecting the most susceptible antibiotic-treated populations. This pathogen is also responsible for approximately 2% of community-acquired diarrhea cases [1, 2]. Only the toxigenic strains that generate toxins A and B have been linked to disease, of which Tcd B is considered to be the main mechanism of CDI. In recent years, the incidence of *C. difficile*-induced diarrhea hospitalizations has increased in many countries, which may be due to the spread of highly virulent strains [3, 4]. In the United States, the incidence of CDI increased from an average of 7.4 per 1,000 adult discharges in 2003 to 13.5 per 1,000 adult discharges in 2012. This rate has continuously increased by approximately 2% annually (e.g., 13.8% in 2013 and 14.4% in 2014) [4]. It is estimated that there are over 500,000 new CDI patients in the US per year, of which approximately 14,000-20,000 cases resulted in death [5-7]. In the United Kingdom, the average CDI occurrence rate has been even higher than in the US, with 8.8 and 15.0 per 1,000 discharged adults in 2003 and 2012, respectively. Approximately 3,000 CDI-associated deaths are reported in the UK each year [4].

In order for patients to receive proper therapy and prevent the spread of infection, accurate and rapid methods for CDI detection are vital. Inpatients that have been infected with toxigenic *C. difficile* strains may remain free of symp-
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toms [8]. Thus, the identification of toxigenic strains in stool samples of symptomatic patients is not enough to identify the cause of diarrhea. Instead, the amount of *C. difficile* toxin in feces might be more descriptive of CDI severity than identifying toxigenic isolates (toxigenic culture, TC) from bacterial cultures. Cell cytotoxicity assays (CCTAs) are considered the “gold standard” for *C. difficile* toxin testing (primarily toxin B) in feces [9]. However, this assay requires between 24 and 48 h to render results. Alternatively, commercial enzyme immunoassays (EIAs) and lateral-flow assays for toxin A and B identification are widely employed. Nevertheless, these tests exhibit significant differences in performance [10, 11].

Given that CDI has a low prevalence in the healthcare sector, commercial tests produce strong negative predictive values (NPVs) in a community, and their positive predictive values (PPVs) are often unexpectedly low [12, 13]. These tests incorporate toxin-targeting EIAs and the identification of *C. difficile* surface-exposed enzymes such as glutamate dehydrogenase (GDH), as well as nucleic acid amplification assays (NAATs) that detect the toxin A or B genes [10, 14-16]. The GDH EIAs and NAATs are more sensitive to bacterial cultures and TCs [14-18].

Although these tests are not suitable for use as standalone diagnostic tools, the UK Ministry of Health guidelines propose a two-step approach, which begins with a screening test consisting of the GDH immunoassay or a NAAT, followed by a sensitive toxin immunoassay for reanalysis of positive specimens. This combination can produce excellent PPVs due to the greatly enhanced pre-test possibilities, which may be highly correlated with disease due to the integration of toxin detection [19-24].

Here, a one-step magnetic-particle-based chemiluminescent enzyme immunoassay (CLEIA) was developed to detect toxin B in feces. The results obtained with this CLEIA showed no significant differences with a commercially available ELISA kit, highlighting the outstanding precision and accuracy of the proposed approach. Compared with ELISA, the newly developed CLEIA method can process large quantities of clinical samples faster, which establishes a good foundation for the replacement of EIAs in the future.

### Materials and methods

#### Reagents

A standard Toxin B dilution series was prepared in 0.01 M phosphate buffer saline (PBS, 0.01 mol/L prepared with KH$_2$PO$_4$ and Na$_2$HPO$_4$, pH 7.4) to reach the target concentrations of 500, 356, 128, 64, 32, 16, 8, 4, and 2 ng/ml assigned to samples S9 to S1, respectively, where 0 ng/ml was assigned to S0 as reference. Bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and complete and incomplete Freund's adjuvants were acquired from Sigma (St. Louis, USA). AMPPD (4-methoxy-4-(3-phosphatephenyl)-3-ethylcarbodiimide hydrochloride (EDC), and complete and incomplete Freund's adjuvants were acquired from Guangzhou Wondfo Biotech Co., Ltd. (Guangzhou, China). Carboxyl micromagnetic particles (6 mm) and acridinium ester (AE) were obtained from Darui Co., Ltd. (Guangzhou, China). Goat anti-rabbit IgG (secondary antibody) were bought from Boster Biotech Co., Ltd. (Wuhan, China). Tcd B monoclonal antibodies were produced in our laboratory. A commercial human *C. difficile* toxin B (CDT) ELISA Kit was supplied by Beijing Huabo Deyi Biotechnology Co., Ltd. (Beijing, China). Human stool samples were supplied by the Southern Medical University. Pre-trigger and trigger liquids were acquired from Darui Co., Ltd. (Guangzhou, China). The PB buffers (pH 6.3) were prepared by dissolving 8.1 g Na$_2$HPO$_4$.12H$_2$O and 12.1 g NaH$_2$PO$_4$.2H$_2$O into 1 L water. For use in ELISA, solution A contained 1 mol/L zinc acetate dihydrate solution and 30 mL of glacial acetic acid per L and solution B was made with 0.25 mol/L potassium ferrocyanide trihydrate. The TBST buffers were prepared by dissolving 0.6 g Tris-HCl, 1.8 g NaCl, 4.0 g BSA, 0.1 g Na$_3$N, 2.0 g trehalose and 100 mL Tween-20 in 200 mL water and adjusted to pH 7.2 with HCl. The binding buffers were prepared by dissolving 9.8 g MES in 500 mL water; the pH was adjusted to 5.0 with a NaOH solution. Double distilled water was used to prepare the solutions.

#### Devices

An automatic chemiluminescent immunoassay system (Limiray 1200, Rayto Life Science Co., Ltd., Shenzhen, China) was used. Antibody concentrations were determined with a NanoDrop (2000c) spectrophotometer (Thermo Fisher Scientific; Shanghai, China). Antibody affinity

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was quantified with a BLItz® system (PALL ForteBio LLC; Menlo Park, USA).

**Tcd B antigen preparation**

The genes coding for the Tcd B fragments were amplified from chromosomal *C. difficile* DNA via polymerase chain reaction (PCR). The overlapping PCR products were cloned into the pHis1525 vector (MoBiTec; Gottingen, Germany) applying the restriction sites BsrGI and KpnI. The transformed *E. coli* colonies were transferred to 100 mL of LB broth incorporating 10 μg/mL tetracycline and cultivated overnight at 37°C with 250 rpm. This culture was used for recombinant protein isolation via C-terminal His6 tags.

Protein purification was conducted through Ni²⁺ affinity chromatography. *B. megaterium* pellets were suspended in 5 mL 20 mM phosphate sodium buffer containing 500 mM NaCl and 30 mM imidazole (pH 7.4). The cells were lysed by sonication and the lysate was centrifuged at 15,500 × g for 30 min at 4°C. The supernatant was then passed through a nickel-charged HisTrap HP column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and the bound protein was eluted with 20 mM phosphate buffer containing 500 mM NaCl and 500 mM imidazole (pH 7.4). The proteins were dialyzed into 20% glycerol PBS buffer and maintained at -80°C [25].

**Preparation and identification of anti-Toxin B monoclonal antibody**

Six eight-week-old BALB/c female mice were bought from the Experimental Animal Center of Nanjing Medical University (Nanjing, China) and were acclimatized for one week before immunization. They were then injected subcutaneously with 100 µg of immunogen emulsified with an equivalent volume of Freund’s complete adjuvant (FCA) for the initial immunization. Afterward, booster immunizations with 50 µg of immunogen emulsified with an identical volume of Freund’s incomplete adjuvant (FIA) were administered every two weeks. Seven days after the second and third treatment, antibody titers were assessed in serum samples obtained from the tail vein with ELISA. The monitoring of hybridoma development and identification of monoclonal antibodies were performed as described by Hongo et al. [26]. We also assessed antibody affinity according to a protocol supplied by the manufacturer [26].

**Coating MPs with Tcd B antibody**

The high-affinity Tcd B monoclonal antibody was covalently coupled to magnetic particles (MPs) in the binding buffer through the terminal amine. First, 2 mL of 20 mg/mL MPs were placed in an Eppendorf tube and washed five times with binding buffer (EDC-NHS). The supernatant was then removed by placing the tube in a magnetic concentrator. After washing, the MPs were resuspended in 2 mL of binding buffer, after which the antibody solution was added and the mixture was incubated at 37°C and mixed overnight to allow the Tcd B antibodies to bind to the MPs. Afterward, the tubes were transferred to a magnetic concentrator to extract the supernatant. Then, 3% BSA was gently shaken and incubated at 37°C for 2 h to block any residual binding sites on the MPs [27]. The coated MPs (mAb-MPs) were then washed five times and allowed to diffuse in 2 mL buffer and stored at 4°C.

**AP-conjugated toxin B antibody preparation**

AP (alkaline phosphates) and anti-Toxin B antibodies specific to different epitopes were coupled with glutaraldehyde, as described below. AP and anti-Toxin B antibodies were diluted in ultrapure water at concentrations of 4 mg/mL and 8 mg/mL, respectively. Equal volumes (250 μL) of these solutions were mixed and 0.5 mL of 1% glutaraldehyde in PBS was added. The blended solution was shaken for 4 h in the dark at 37°C, after which 0.1 mL of 1 mol/L monoethanolamine was added and the incubation continued for 2 hours at ambient temperature. The blended solution was then dialyzed overnight into PBS solution at 4°C. Upon dialysis, the toxin B antibody-AP conjugate was transferred to an Eppendorf tube and mixed with an equivalent amount of glycerol and 1% BSA. These AP-conjugated toxin B antibodies (AP-mAbs) were preserved at -20°C for downstream experiments.

**MP-based fully-automated CLEIA**

Tcd B was tested using a fully-automated chemiluminescent immunoassay (Limiray 1200) based on a sandwich-type immunoassay for anti-Tcd B antibody-coated MPs and AP-labeled anti-Tcd B antibodies, both of which identify various Tcd B epitopes. The antibody-coated MPs replace the solid phase of microplates in conventional microplate assays. The entire
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immunoassay process was completed using a completely automated chemiluminescence immunoassay analyzer. Figure 1 illustrates a schematic of the method. Approximately 50 μL of mMAb-MPs was mixed with 30 μL of Tcd B at different concentrations at 37°C for 20 min (capture time) under gentle oscillation (i.e., mixing). Non-specific binding was prevented by washing three times with a PBS solution containing 0.05% Tween-20 in a magnetic wash station. AP-mAbs anti-Tcd B antibodies were added and incubated for 10 min at 37°C with gentle oscillation, during which an MP-Tcd B-AP sandwich-shaped immune complex was produced. The immune complex was separated magnetically and washed as described above to remove excess AP-mAbs. Afterward, 200 μL of an AMPPD solution including was added to the sandwich complex. The resulting mixture was transferred to an immunoassay analyzer and the relative light unit (RLU) value was evaluated.

Optimization of the immunoassay reagent

Various experimental variables were optimized to obtain the widest testing range (RLUS9/RLUS0) and to achieve optimal sensitivity (RLUS1/RLUS0). The optimal MP-mAbs and AP-mAbs dilutions were 1:50 and 1:100, respectively. Other experimental parameters such as substrate volume, incubation time, optimal pH, stability, and precision were studied as well. To evaluate the feasibility of the proposed immunoassay, we analyzed 104 specimens with our proposed immunoassay as well as with a commercial ELISA kit.

Results and discussion

Production and identification of mAb against toxin B

Two mAbs (C6, E6) specific to different toxin epitopes were purified. The two antibodies were tested in a sandwiched ELISA assay as described in the Methods. C6 was employed as a capturing antibody and E6 as the detecting antibody. Given that the antibody purity could impact the specificity and sensitivity of the assay, the antibodies were analyzed via SDS-PAGE electrophoresis and were found to be > 85% pure (Figure 2). Each mAb appeared as two visible bands representing the heavy and light chains respectively, and all other proteins were almost completely removed.

Immuonassay optimization

Optimization of AP-mAbs and mAb-MPs concentration: The quantities of mAb-MPs and AP-mAbs antibodies are the variables that most affect the accuracy and sensitivity of an
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Optimization of the substrate buffer pH: Optimal pH and the incubation time were determined due to the importance of these parameters; pH significantly influences the activity of immobilized proteins, whereas excessively long incubation times can result in dissociation of the antigen-antibody complex. The two variables were evaluated by identifying maximum RLUs for the immunoassay performed with a toxin B standard sample (S3, 8 ng/mL). The mAb-MPs solution (1:50, 50 µL), AP-mAbs solution (1:100, 50 µL), and the standard sample (S9, 30 µL) were incubated at 37°C for 0-60 min. During this incubation, the MPs-toxin B-AP immunocomplex was washed every 5 minutes and chemiluminescent substrates (200 µL) at various pH values were used to assess the RLU. The pH of the substrate buffers was

Figure 2. Purification results of the mAb against Tcd B by SDS-PAGE. Only two bands were identified after the mAb was purified, and these corresponded with heavy and light chains, respectively. The results indicate that the purity of all mAbs was above 85% as analyzed by SDS-PAGE.

Figure 3. AP-mAb and mAb-MP concentration optimization. Here, the dilution ratios of mAb-MPs and AP-mAbs were optimized by analyzing a series of dilution ratios with standard positive samples (S3, 8 ng/mL) and negative samples (S0, 0 ng/mL). Likewise, the RLUS3/RLUS0 signal ratio was used as an indicator for the identification of the optimal assay. Figure 3 shows that the RLUS3/RLUS0 signal ratio increased when the dilution ratios of AP-mAbs changed from 1:500 to 1:100 for each dilution ratio of the mAb-MPs examined herein (P<0.05). However, with AP-mAbs dilution ratios of 1:50 and 1:100, the RLUS3/RLUS0 discrepancy was inconsequential. In mAb-MPs, the RLUS3/RLUS0 ratio peaked at a dilution ratio of 1:50 (P<0.05). As the amounts of mAb-MPs decreased from 1:50 to 1:400, RLUS3/RLUS0 gradually increased, suggesting that the available Tcd B was not captured completely. When mAb-MPs at a 1:50 ratio were added, there was an adequate amount of antigen to bind to mAb-MPs, leading to an optimal RLUS3/RLUS0 ratio. When mAb-MPs were added at a ratio exceeding 1:50 (1:20), excessive MAb-MPs resulted in a lowered sensitivity due to tightly gathered mAb-MPs particles, resulting in absorption of the emitted light and luminescence blockage. Therefore, given the test sensitivity and cost, further experiments were conducted using AP-mAbs and mAb-MPs with dilution ratios of 1:100 and 1:50, respectively.
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![Figure 4](image1.png)

**Figure 4.** Optimization of substrate buffer pH and incubation time. The maximum RLU value was obtained at a pH of approximately 9.0 and was therefore considered the optimal pH value for our diagnostic test. Regarding the incubation time, the maximal RLU increased with incubation time from 0 to 30 min and was stable between 30 to 50 min, demonstrating that the antigen-antibody complex formation had reached equilibrium. Therefore, the optimal incubation time was found to be 30 min.

![Figure 5](image2.png)

**Figure 5.** Tcd B dose-response calibration curve obtained from fully-automated CLEIA. A series of Tcd B standard samples were used (n = 3). The black circle indicates the detection value of each standard concentration; the error bar indicates the standard deviation; the black straight line connects the standard detection values used.

adjusted with a 1 M NaOH solution. **Figure 4** illustrates the influence of pH and incubation time on overall light emission kinetics. The RLU intensity increased progressively until a steady state was obtained. The biphasic behavior caused by the comprehensive light emission dynamics resulted from a two-step process that led to emission delay prior to chemiluminescence in a steady state. The RLU value increased from 0 to 20 minutes at pH 7.0 and formed an unstable plateau at longer reaction durations. Moreover, the maximum RLU at pH 7.0 was lower than that at other pH conditions. At a pH of approximately 9.0, the RLU achieved a maximum and was therefore considered the optimal pH value for our diagnostic test; these findings were almost identical to those reported in a previous study [28]. Regarding the incubation time, the maximal RLU increased with incubation time from 0 to 30 min and was stable between 30 to 50 min, demonstrating that the antigen-antibody complex formation had reached equilibrium. Therefore, the optimal incubation time was found to be 30 min.

**Method evaluation**

**Calibration curve for toxin B identification:** Once the reaction conditions had been optimized, a concentration series of standard Tcd B solutions (S0-S7, 0-128 ng/mL) was measured. A calibration curve was constructed with toxin B standards (**Figure 5**), where the absorbance limit of detection (LOD) was calculated by adding two times the average of the standard deviation of 10 S0 wells. This resulted in a detection range from 0.12 to 150 ng/mL with a LOD of 0.49 ng/mL and a correlation coefficient of 0.9997. These results indicated that the fully-automated MMPs-based CLEIA proposed herein exhibited a better clinical diagnosis performance than typical commercial ELISA kits.

**Accuracy, precision, and stability analyses:** Dilution recovery rates were examined to evalu-
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Cross-reactivity of Tcd B immunoassay: Cross-reactivity between Tcd B, Tcd A, and BSA was then evaluated for our immunoassay. The RLU values were determined for the analyte, and the cross-reactivity (CR) for each analog was calculated. Our CLEIA test showed high specificity for the detection of Tcd B with no significant cross-reactivity even at very high concentrations of analytes and Tcd B antibodies (Table S2).

Conclusions

In summary, we proposed a micromagnetic-particle-based chemiluminescent immunoassay and tested it by identifying toxin B in stool samples. This technique was also implemented to identify toxin B in clinical stool samples, which rendered comparable results to those of a commercial toxin B detection ELISA kit. However, unlike the conventional microplate ELISA, our proposed assay had three outstanding properties: (1) the large surface-to-area ratio of MMPs contributes to more response sites; (2) the implementation of an automatic chemiluminescent immunoassay significantly reduces the assay time to less than 40 minutes; (3) the accuracy of the method. Five stool specimens were linearly diluted (up to 1:32) and tested thereafter (Figure 6). The product of the measured results and the dilution factor was compared with results obtained with the undiluted concentrations. The deviation caused by dilution remained below 15% in all cases. To determine the precision of the approach, intra-assay and inter-assay comparisons were conducted with three toxin B standard concentrations; intra-assay and inter-assay experiments were replicated 6 times each (the latter were performed on different days). Table 1 shows that the two coefficients of variation (CVs) were below 15%. Further, the stability of the fully-automated CLEIA was explored. After keeping the mAb-coated MPs at 4°C and toxin B standards and AP-labeled mAb at -20°C for 30 days. No significant differences were identified in the test values after storage for 10, 20, and 30 days (Table S1). This result highlighted the high stability of our fully-automated CLEIA method.

Analysis of spiked samples and blind samples: Table 2 shows that the average recoveries ranged from 97.3% to 103.25%. To evaluate the efficiency of the analytical approach, we analyzed 104 stool samples and compared the results with a commercial ELISA kit. As shown in Figure 7, the results from the two approaches were consistent. The resulting linear regression equation obtained was \( Y = 0.9984*X + 0.4420 \) (\( R^2 = 0.9893 \)). The results indicate that the newly developed assay served as a more competitive alternative for the clinical determination of toxin B in human stool samples.

### Table 1. Intra-assay and inter-assay tests

<table>
<thead>
<tr>
<th>Times</th>
<th>Added toxin B concentration (ng/mL)</th>
<th>20.0 (n = 6)</th>
<th>40.0 (n = 6)</th>
<th>80.0 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD</td>
<td>CV (%)</td>
<td>Average</td>
</tr>
<tr>
<td>1</td>
<td>19.86</td>
<td>0.36</td>
<td>3.70</td>
<td>39.14</td>
</tr>
<tr>
<td>2</td>
<td>19.92</td>
<td>0.28</td>
<td>2.85</td>
<td>39.94</td>
</tr>
<tr>
<td>3</td>
<td>19.94</td>
<td>0.39</td>
<td>4.01</td>
<td>39.69</td>
</tr>
<tr>
<td>4</td>
<td>20.25</td>
<td>0.65</td>
<td>3.29</td>
<td>39.93</td>
</tr>
<tr>
<td>5</td>
<td>20.38</td>
<td>1.28</td>
<td>6.30</td>
<td>41.08</td>
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<tr>
<td>6</td>
<td>20.03</td>
<td>0.59</td>
<td>2.92</td>
<td>40.51</td>
</tr>
</tbody>
</table>

### Table 2. CLEIA analyses of a toxin B standard dilution curve

<table>
<thead>
<tr>
<th>Tcd B concentration (ng/mL)</th>
<th>Spiked concentration (ng/mL)</th>
<th>Mean measured concentration (ng/mL)</th>
<th>Mean recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.76</td>
<td>5</td>
<td>6.98</td>
<td>103.25</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>31.15</td>
<td>98.1</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>79.55</td>
<td>97.3</td>
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<td>5.12</td>
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<td>10.39</td>
<td>102.26</td>
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<td></td>
<td>30</td>
<td>36.16</td>
<td>102.96</td>
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<td>85.89</td>
<td>100.9</td>
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<tr>
<td>21.47</td>
<td>5</td>
<td>25.89</td>
<td>97.8</td>
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<td>30</td>
<td>51.15</td>
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</tr>
<tr>
<td></td>
<td>80</td>
<td>100.52</td>
<td>98.67</td>
</tr>
</tbody>
</table>
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Figure 7. Comparison between CLEIA and ELISA for clinical Tcd B detection. The results from the two approaches were consistent. The resulting linear regression equation obtained was $Y = 0.9984X + 0.4420$ ($R^2 = 0.9893$). The results indicate that the newly developed assay served as a more competitive alternative for the clinical determination of toxin B in human stool samples.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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[12] Code P. Clostridium difficile toxins Other toxins Antibodies directed against Clostridium difficile Antibodies directed against Salmonella spp. Antibodies directed against EHEC/EPEC bacteria. 2018; 49.


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**Table S1.** Stability of the reagents (n = 3)

<table>
<thead>
<tr>
<th>Tcd B (ng/ml)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>RD (%)</td>
<td>Value</td>
<td>RD (%)</td>
</tr>
<tr>
<td>10.5</td>
<td>10.2±0.09</td>
<td>1.11</td>
<td>10.54±0.64</td>
</tr>
<tr>
<td>20.4</td>
<td>19.66±0.18</td>
<td>-2.76</td>
<td>21.86±1.31</td>
</tr>
<tr>
<td>39.8</td>
<td>38.97±0.5</td>
<td>0.14</td>
<td>40.38±0.87</td>
</tr>
<tr>
<td>101.97</td>
<td>98.94±0.71</td>
<td>1.46</td>
<td>99.73±0.21</td>
</tr>
</tbody>
</table>

**Table S2.** Cross-reactivity of CLIA to related compounds

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Added concentration</th>
<th>RLU × 1000</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tcd B</td>
<td>10 ng/ml</td>
<td>32.014</td>
<td>100%</td>
</tr>
<tr>
<td>Tcd A</td>
<td>100 ng/ml</td>
<td>0.17</td>
<td>0.53%</td>
</tr>
<tr>
<td>BS A</td>
<td>100 ng/ml</td>
<td>0.02</td>
<td>0.06%</td>
</tr>
</tbody>
</table>