**Clostridium difficile** Infection Is More Severe When Toxin Is Detected in the Stool than When Detected Only by a Toxigenic Culture

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**Abstract**

**Objective**  *Clostridium difficile* infection (CDI) is the major cause of antibiotic-associated diarrhea in hospital inpatients. Rapid testing for the toxins in stool specimens is inconclusive due to its low sensitivity. Therefore, a two-step method is recommended as the most appropriate approach. The purpose of the present study was to evaluate the differences in the disease severity score between the patients who were glutamate dehydrogenase (GDH)-positive/enzyme immunoassays (EIA) toxin-positive (group A) and those who were GDH-positive/EIA toxin-negative, but who were nonetheless finally confirmed to be toxin-positive by toxigenic culture testing (group B).

**Methods**  A rapid detection EIA for GDH and toxin A/B were simultaneously performed for initial screening. Subsequently, the toxin production by bacterial colonies in culture was retested with the same rapid test kit when necessitated by an equivocal result of the initial screening.

**Results**  A total of 334 fecal specimens were evaluated. Group A consisted of 25 specimens (from 16 patients) and group B consisted of 27 specimens (from 12 patients). The severity score (based on a number of factors, including age, body temperature, serum albumin level and white cell count) of group A and B was 2.2±0.7 and 1.4±0.5, respectively (p=0.002).

**Conclusion**  The cases of CDI in which the toxins were detected by the initial screening test were more severe than those where the toxins were not detected at the initial screening but were identified by the toxigenic culture. In addition, the most significant factors affecting the severity score were an older age and a lower serum albumin level.

**Key words:** *Clostridium difficile*, toxin, glutamate dehydrogenase, two-step method


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**Introduction**

*Clostridium difficile* is the most common causative microorganism for nosocomial diarrhea, accounting for 15-25% of antibiotic-associated diarrhea \(^1,2\). The clinical manifestations of toxin-producing *Clostridium difficile* infection (CDI) range from mild or moderate diarrhea to fulminant or fatal enteritis, including pseudomembranous colitis, ileus, toxic megacolon and intestinal perforation.

In addition, CDI is an important hospital-associated infection. The cumulative mortality rate related to CDI ranges from 5.5% to 6.9%, with a higher mortality during severe outbreaks \(^3\). It is a spore-forming anaerobe resistant to alcohol. As alcohol is globally used as a skin disinfectant, it is possible for the pathogen to be readily transmitted from one person to another in a health-care setting. Nosocomial spread burdens the hospital with excessive health-care costs; the economic costs of CDI were estimated to be high for both primary and recurrent cases in a recent systematic review \(^4\). Therefore, a rapid and accurate diagnosis is crucial in consideration of both the medical treatment and infection

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control. The patients presenting with diarrheal or unformed stool after hospitalization for three or more days should be tested for CDI immediately if the cause is uncertain.

There are many different methods of testing, each of which has advantages and disadvantages. Thus, making a diagnosis of CDI more efficiently and effectively remains a challenge to the clinician and the microbiologist (5). In concordance with various different studies, a two-step method is recommended as the most appropriate approach (2). In our institution, the laboratories perform rapid detection enzyme immunoassay (EIA) tests for glutamate dehydrogenase (GDH) and toxin A/B simultaneously as an initial screening test. Positive results of both are accepted as truly positive, and reciprocally when both are negative. However, when the GDH test is positive but the toxin test is negative, there is a possibility of a false negative due to low sensitivity. Consequently, the toxin production of cultured bacteria is retested with the same rapid test kit.

One problem with this approach is that it takes several days for the final confirmatory test when GDH is positive but EIA toxin is negative. This causes a potential risk by delaying the initiation of appropriate therapy. However, to the best of our knowledge, no studies comparing the clinical grade of severity between GDH-positive/EIA toxin-positive and GDH-positive/EIA toxin-negative patients where the final confirmation of toxin-positivity is obtained by a toxigenic culture are currently available.

Because there are no studies indicating that delaying the initiation of therapy is permissible, we herein evaluate the clinical manifestations and laboratory test results of diagnosed CDI using the recommended two-step algorithm and determine the differences between the GDH-positive/EIA toxin-positive cases and those cases which were initially GDH-positive/EIA toxin-negative but ultimately confirmed to be toxin-positive by the toxigenic culture.

Materials and Methods

Samples and patients

All patients with CDI diagnosed between April 1, 2013 and March 31, 2014 in Fujisawa City Hospital, Japan were included in the present study. A total of 334 fecal specimens were collected from the patients with symptoms of diarrhea. Diarrhea was defined as an increased frequency of defecation ≥3 times per day or looser stool than usual.

Detection of GDH and toxin A/B

All stool specimens were subjected to an immunochromatographic assay for GDH and toxin A/B simultaneously by C. DIFF QUIK CHEK COMPLETE (Alere Medical, Tokyo, Japan) in accordance with the manufacturer’s instructions. The results were classified into three patterns as follows: GDH-positive/EIA toxin-positive, GDH-negative/EIA toxin-negative and GDH-positive/EIA toxin-negative. In the latter instance, retesting using three to five bacterial colonies cultured on cycloserine-cefoxitin mannitol agar (CCMA) for 48 hours was routinely carried out.

Severity grade of CDI

The severity of symptoms in each patient was scored as previously described (6). Severe infection was defined according to the presence of pseudomembranous colitis on endoscopy, admission to an ICU, or any two of the following: age >60 years, temperature >38.3°C, a serum albumin level <2.5 g per deciliter, and a white-cell count >15,000 cells per cubic millimeter. We estimated the severity grade according to the summed number of any of the above factors. After the case identification, complete medical records were reviewed and the laboratory data were collected retrospectively from the patients’ electronic medical charts.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows software program. Data were expressed as the mean ± standard deviation (SD). In all the cases, p values <0.05 from a two-tailed test were considered to be statistically significant.

Results

During the one-year study period, a total of 334 fecal specimens were collected and tested for GDH and toxin A/B (Figure). Of these, 252 specimens were GDH-negative/EIA toxin-negative, indicating no CDI. There were no GDH-negative/EIA toxin-positive cases. The remaining 82 GDH-positive specimens fell into two groups depending on whether or not EIA toxin A/B was detected. Of these specimens, 25 were GDH-positive/EIA toxin-positive, yielding an unequivocal diagnosis; however, 57 specimens were GDH-positive/EIA toxin-negative, thus with an uncertain diagnosis. The group with toxin detected in the stool consisted of 16 patients (group A). The latter EIA toxin-negative specimens were cultured under anaerobic conditions on CCMA for approximately 48 hours at 37°C and retested using the bacterial colonies, except for 7 cases which could not be cultured despite being GDH-positive. Of the remaining specimens, 27 were EIA toxin-positive (from 12 patients; group B) whereas the other 23 remained EIA toxin-negative. CDI was subsequently considered to be confirmed by the evidence of the toxin production. Despite the presence of GDH, negativity for EIA toxin, even in the toxigenic culture, implies that in these patients the bacterial strains do not produce any toxins, or so little that it is under the level of detection.

We compared group A (16 patients) with group B (12 patients) in terms of the patients’ characteristics and risk factors associated with a causative relationship to CDI, treatment, recurrence rate and severity score (Table).

The mean age was 77.0±8.7 years in group A and 64.6±22.0 years in group B (p=0.049). CDI was more prevalent in
Figure. Flow chart for the three-step approach to the diagnosis of *Clostridium difficile* infection. Our laboratory performs immunochromatographic assays for GDH and toxin A/B simultaneously by C. DIFF QUIK CHEK COMPLETE, which enables us to conduct the first and second steps at the same time. When the result is GDH-positive/EIA toxin-negative, we subsequently assess the toxin production of cultured bacterial colonies retested with the same rapid test kit in the third step.

There were no cases of pseudomembranous colitis as established by endoscopy or on admission to the ICU. The mean body temperature was 37.8±1.2°C in group A and 37.9±1.2°C in group B (p=0.846). The white blood cell count was 11.081±6.538/μL and 8.500±5.528/μL in groups A and B, respectively (p=0.280). The C-reactive protein level was 9.3±8.6 mg/dL in group A and 5.3±4.4 mg/dL in group B (p=0.159). Conversely, the serum albumin level was 2.5±0.5 g/dL in group A and 3.1±0.8 g/dL in group B (p=0.024). Consequently, the severity score consisting of these four parameters was 2.2±0.7 in group A and 1.4±0.5 in group B (p=0.002).

### Discussion

In the present study, we evaluated the clinical severity of CDI between two groups of patients who were either GDH-positive/EIA toxin-positive according to the initial direct screening stool specimens or who were GDH-positive and positive for EIA toxin only by a toxigenic culture, not by the direct stool measurement. The clinical state of the cases of CDI where the toxins were detected in the initial direct screening test (i.e., GDH-positive/EIA toxin-positive) was more severe than in the patients with CDI where the toxins were only after the toxigenic culture.

Many different testing methods exist to assist the diagnosis of CDI, including EIA for toxin A or toxin A and B, EIA for GDH, polymerase chain reaction (PCR) testing, cell cytotoxicity assays and toxigenic cultures. Most laboratories perform EIA for toxin A or toxin A and B, but in general this method has a low sensitivity (as little as 63% to as high as 94% in some studies), with a specificity of 75-100% (2). PCR testing appears to be rapid, accurate, sensitive and specific; however, it imposes higher initial costs due to the costs of necessary equipment, thereby making it less feasible for routine testing in every institution. A cytotoxicity assay also requires special instruments and techniques, making it unfeasible for many laboratories despite the fact that such assays are traditionally the gold standard method for detecting toxins. Therefore, the optimal strategy for providing accurate, prompt, cost-effective results has remained controversial over a long period of time (5).

One strategy to resolve these problems is a two-step approach that uses the EIA detection of GDH as an initial screening test followed by a confirmatory cell cytotoxicity assay or toxigenic culture for stool specimens positive for GDH alone (2, 7-10). Many laboratories in Japan, including our institution, have adopted a three-step approach (as shown in Figure). First, we perform an immunochromatographic assay for GDH and toxin A/B simultaneously using C. DIFF QUIK CHEK COMPLETE. This enables us to conduct the first and second steps at the same time. When both the GDH and EIA toxin test are positive, a definitive positive diagnosis is confirmed. Additionally, when both the GDH and EIA toxin test are negative, a definitive negative diagnosis for CDI is confirmed. However, in the present study, 57 specimens were GDH-positive but EIA toxin-negative in the initial screening. We then implemented the 3rd step and found that 27/57 (47.4%) specimens produced toxins in culture. These cases may have otherwise been overlooked and conceivably we may have used standard precautions when providing care for those patients.

We recommended assessing the disease severity score in all CDI patients because this influences the selection of first-line antimicrobial drugs (2, 11, 12). In several protocol recommendations, metronidazole remains the first-line agent for the treatment of a mild infection, however, vancomycin may be recommended as the first-line agent for a more severe infection because of its greater effectiveness and significantly lower risk of treatment failure. However, it should be noted that there are currently no prospective studies of the severity scores for CDI. To the best of our knowledge, there are two severity score systems. One consists of four clinical factors: age, body temperature, the serum albumin level and white cell count (6). The other system consists of two laboratory data: white cell count >15,000 cells per cubic millimeter and a serum creatinine level >1.5 times the
Table. Comparison of the Characteristics of the Patients in the GDH-positive/EIA Toxin-positive Group (group A) and the Patients who Initially Tested GDH-positive/EIA Toxin-negative but were Confirmed to be Toxin-positive by a Toxigenic Culture (group B).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall (n=28)</th>
<th>Group A (n=16)</th>
<th>Group B (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>71.7 ± 16.7</td>
<td>77.0 ± 8.7</td>
<td>64.6 ± 22.0</td>
<td>0.049</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>21 (75.0%)</td>
<td>12 (75.0%)</td>
<td>9 (75.0%)</td>
<td>1</td>
</tr>
<tr>
<td>Malignancy</td>
<td>14 (50.0%)</td>
<td>5 (31.3%)</td>
<td>9 (75.0%)</td>
<td>0.054</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Past prescribed antibiotics</td>
<td>26 (92.9%)</td>
<td>16 (100.0%)</td>
<td>10 (83.3%)</td>
<td>0.175</td>
</tr>
<tr>
<td>Antibiotics cover anaerobes</td>
<td>14 (50.0%)</td>
<td>7 (43.8%)</td>
<td>7 (58.3%)</td>
<td>0.703</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>5 (17.9%)</td>
<td>1 (6.3%)</td>
<td>4 (33.3%)</td>
<td>0.133</td>
</tr>
<tr>
<td>Acid reducing agent</td>
<td>21 (75.0%)</td>
<td>13 (81.3%)</td>
<td>8 (66.7%)</td>
<td>0.418</td>
</tr>
<tr>
<td>PPI</td>
<td>14 (50.0%)</td>
<td>10 (62.5%)</td>
<td>4 (33.3%)</td>
<td>0.252</td>
</tr>
<tr>
<td>H2 blocker</td>
<td>8 (28.6%)</td>
<td>3 (18.8%)</td>
<td>5 (41.7%)</td>
<td>0.231</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>6 (21.4%)</td>
<td>2 (12.5%)</td>
<td>4 (33.3%)</td>
<td>0.354</td>
</tr>
<tr>
<td>Tube feeding</td>
<td>4 (14.3%)</td>
<td>1 (6.3%)</td>
<td>3 (25.0%)</td>
<td>0.285</td>
</tr>
<tr>
<td>Intestinal surgery</td>
<td>7 (25.0%)</td>
<td>3 (18.8%)</td>
<td>4 (33.3%)</td>
<td>0.418</td>
</tr>
<tr>
<td>Days from admission</td>
<td>34.7 ± 69.4</td>
<td>33.0 ± 40.8</td>
<td>36.9 ± 97.8</td>
<td>0.886</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>19 (67.9%)</td>
<td>13 (81.3%)</td>
<td>6 (50.0%)</td>
<td>0.114</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>6 (21.4%)</td>
<td>2 (12.5%)</td>
<td>4 (33.3%)</td>
<td>0.354</td>
</tr>
<tr>
<td>No antibiotics</td>
<td>3 (10.7%)</td>
<td>1 (6.3%)</td>
<td>2 (16.7%)</td>
<td>0.560</td>
</tr>
<tr>
<td>Probiotics</td>
<td>21 (75.0%)</td>
<td>12 (75.0%)</td>
<td>9 (75.0%)</td>
<td>1</td>
</tr>
<tr>
<td>Recurrence</td>
<td>7 (25.0%)</td>
<td>5 (31.3%)</td>
<td>2 (16.7%)</td>
<td>0.662</td>
</tr>
<tr>
<td>Body temperature (degrees C)</td>
<td>37.8 ± 1.2</td>
<td>37.8 ± 1.2</td>
<td>37.9 ± 1.2</td>
<td>0.846</td>
</tr>
<tr>
<td>White blood cell count (µL)</td>
<td>9,975 ± 6,155</td>
<td>11,081 ± 6,538</td>
<td>8,500 ± 5,528</td>
<td>0.280</td>
</tr>
<tr>
<td>C-reactive protein (mg/dL)</td>
<td>7.6 ± 7.2</td>
<td>9.3 ± 8.6</td>
<td>5.3 ± 4.4</td>
<td>0.159</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>2.8 ± 0.7</td>
<td>2.5 ± 0.5</td>
<td>3.1 ± 0.8</td>
<td>0.024</td>
</tr>
<tr>
<td>Severity score</td>
<td>1.9 ± 0.7</td>
<td>2.2 ± 0.7</td>
<td>1.4 ± 0.5</td>
<td>0.002</td>
</tr>
</tbody>
</table>

SD: standard deviation, PPI: proton pump inhibitor

premorbid level). However, the latter criteria proposed for defining severe CDI are based on expert opinion and need to be validated in prospective clinical studies (2). For the reasons mentioned above, we used the first set of criteria in the present study.

The severity score (the number of positive factors) of group A and B was 2.2±0.7 and 1.4±0.5, respectively (p=0.002). The most significant factors which affected the severity score were an older age and a lower serum albumin level. There were no significant differences observed in the serum albumin levels before and after developing diarrhea. Therefore, low albumin levels were not the result of diarrhea but the cause of severe CDI. More specifically, the poorly nourished patient had a greater chance of developing more severe CDI.

Hence, CDI patients with toxins detected by the initial screening test had more severe disease than those where the toxins were first detected by the toxigenic culture. We speculate that this difference was due to the amount of toxin produced. The EIA for toxin have good specificity and have come into widespread use; however, there is a sensitivity issue in that 100 to 1,000 pg of either toxin A or toxin B must be present for the test to be positive (1). Warny et al. reported that the severity of CDI caused by NAP1/027 could result from the hyperproduction of toxin A and B (13). The more toxin the strain produces, the more severe the symptoms will be. Therefore, CDI where the toxins were not detected at the initial screening due to their low level of production but were detected by the toxigenic culture would be expected to be mild, thus a delay of several days before initiating treatment would be permissible for that individual patient. However, we must take into consideration the possibilities for nosocomial spread would be equally great, because of the presence of the pathogen. When GDH tests positive but the EIA toxin is negative, the patient must nonetheless be accommodated as clearly having CDI and contact precautions must be applied before the results of the toxin production tests become available, because in the pre-
sent study, approximately half of these cases were ultimately confirmed to be toxin-producing.

There are several limitations associated with the present study. First, our sample size was small and the study was conducted at a single institution. Second, we did not specifically measure the amount of toxins.

In conclusion, we herein reported that CDI in the patients where the toxins were detected by initial screening tests (GDH-positive/EIA toxin-positive) was more severe than in the cases where the toxins were not detected at screening but only after a toxigenic culture. In addition, the most significant factors which affected the severity score were an older age and lower serum albumin level.

The authors state that they have no Conflict of Interest (COI).

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References


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