Does the handling time of unrefrigerated human fecal specimens impact the detection of *Clostridium difficile* toxins in a hospital setting?

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Abstract

**Background** The stability of *Clostridium difficile* toxins is an important factor in determining the accuracy of the enzyme immunoassay (EIA). The Centers for Disease Control has stated that *C. difficile* toxins may become undetectable in unrefrigerated stool specimens within 2 h after collection.

**Purpose** The purpose of our study was to determine how the unrefrigerated handling time of human fecal specimens affects the results of *C. difficile* infection (CDI) testing.

**Methods** A retrospective review of CDI testing with Premier™ Toxins A and B kit was conducted in northern New Jersey, USA. Stool collection times and receiving times were recorded for each specimen. The unrefrigerated handling time was calculated for each.

**Results** A total of 1126 fecal specimens were submitted. We excluded 72 fecal specimens due to incomplete documentation. We included 1054 fecal specimens collected from 636 hospitalized patients. A total of 132 out of 1054 specimens (12.5%) tested positive for *C. difficile* toxins. Nine hundred and fifty-four specimens were unrefrigerated for 13 h or less, of which 127 (13.3%) tested positive. Five (5%) of the 100 specimens that were unrefrigerated for more than 13 h tested positive (*p*=0.02).

**Conclusion** *C. difficile* toxins can still be detected up to 13 h after collection in unrefrigerated human fecal specimens. However, fecal specimens should be processed according to the current recommendations to ensure the reliability of EIA testing until the results of our study are confirmed with prospective studies.

**Keywords** Handling time · Human fecal specimen · Toxin A and B stability

Introduction

A number of laboratory tests are available to diagnose *Clostridium difficile* infection (CDI) [1]. The most widely used test is the EIA [1, 2], which is 88–93% sensitive and 94–100% specific [2]. Rapid diagnosis with immunoassays followed by prompt medical therapy results in high cure rates in CDI [3]. Hence, commercially available EIA is commonly used in the clinical setting [1, 2]. EIA accurately detects toxin A and toxin B in fecal specimens [1, 2, 4–6], is cost-effective, easy to perform, and offers the fastest turnaround time of minutes to hours [1–6].

*C. difficile* is an obligate anaerobe [7, 8]. However, fecal specimens are not typically stored in an anaerobic environment. The vegetative cells of *C. difficile* survive from 15 min [8, 9] to 2 h [10] in aerobic conditions. Moreover, *C. difficile* toxins are also unstable [8, 11–13] and as a
result, their stability in fecal specimens can affect clinical diagnosis [8]. Bowmen and Riley found that there was a 1.7 and a 1.4 log reduction in average fecal C. difficile cytotoxin titers after 2 days of storage at 25°C (room temperature) and 5°C, respectively [14]. However, the clinical significance of their finding is unclear because it involved only 3 specimens. Studies evaluating horse fecal specimens have suggested that unrefrigerated fecal specimens should be tested within 3–4 h of collection [15]. According to the Centers for Disease Control (CDC), Atlanta, U.S.A., the toxin degrades at room temperature and may become undetectable within 2 h after collection of a fecal specimen [11]. False negative results may occur if unrefrigerated specimens are not tested immediately after collection [8, 11]. None of these observations have been confirmed in large scale studies [8]. The purpose of our study was to determine how the handling time of unrefrigerated human fecal specimens affects the results of CDI testing using EIA.

Methods

Study design

A retrospective review examining the results of CDI testing using Premier™ Toxins A and B kit (Meridian Bioscience, Inc., Cincinnati, Ohio, U.S.A.) was conducted at a medium-sized, 357-bed, inner-city hospital in northern New Jersey, U.S.A.

Data collection

The collection time (the time of collection of specimens by nursing staff), receiving time (the time each specimen was received in the laboratory), and result of fecal specimens sent for CDI testing from hospitalized patients between December 1, 2008 and July 31, 2009 were reviewed. We included the results of all the fecal specimens which had documented collection and receiving times. The handling time (defined as the time interval between collection time and receiving time) was calculated for each specimen. All fecal specimens were tested for CDI using Premier™ Toxins A and B kit.

Specimen collection and testing

The fecal specimens were collected by the nursing staff and sent to the microbiology laboratory for analysis. The patient identification number and specimen collection time for each specimen were documented by the nursing staff. The specimens were not refrigerated and kept at room temperature (20–25°C) until they arrived in the laboratory. Nursing staff, floor technicians, and an electronic pneumatic suction channel were used to transport the fecal specimens. As soon as the specimens reached the laboratory receiving area, the receiving time was documented and entered into the computer system by the microbiology laboratory technologists. The specimens were immediately refrigerated at 2° to 8°C. The refrigerated specimens were processed in batches. Laboratory technologists in consultation with a microbiologist processed the specimens and ran the tests for the presence of C. difficile toxin A and B. Results were recorded (positive or negative) in the laboratory computer system.

Biological principles

Premier™ Toxins A and B is a qualitative enzyme immunoassay for the detection of C. difficile toxin A and toxin B in fecal specimens from patients with antibiotic-associated diarrhea. A positive result indicates the presence of C. difficile toxin A or B or both. A negative result indicates the absence of toxins A and B or that the level of toxin is below that which can be detected by the assay (Premier™ Toxins A and B, Enzyme Immunoassay kit).

Statistical analysis

Statistical analysis was done using SPSS 16 Statistical Software, SPSS Inc. Specimen results (positive or negative) and a handling time groups were compared using χ² tests. Mean times were evaluated using a Mann-Whitney U test. An alpha of 0.05 was considered significant.

Results

A total of 1126 fecal specimens were submitted to the hospital laboratory over an 8 month period. We excluded 72 fecal specimens from 35 patients due to incomplete documentation of collection and or receiving times. We included the remaining 1054 fecal specimens collected from 636 hospitalized patients. A total of 132 out of 1054 specimens (12.5%) tested positive for C. difficile toxin. The range of the handling time for all specimens was 30 min to 78.5 h (median [interquartile range] 212.5 [116.0–452.0] minutes). The range of handling time for C. difficile toxin negative fecal specimens was 32 min to 78.5 h (median [IQR] 211 [116.75–469.0] minutes). The range of handling time for C. difficile toxin positive fecal specimens was 30 min to 20 h (median [IQR] 217.5 [113.25–357.75] minutes). Using the Mann-Whitney U test there was no difference between the handling time of the specimens that had a positive result (M rank=512.27, n=132) compared to those that had a negative result (M rank=529.68, n=922, z=−0.615, p=0.54). Among the 132 positive specimens, 96
(72.7%) had a handling time of more than 2 h. The percentages of positive results for each hourly grouping are demonstrated in Fig. 1.

Thirty-six out of 283 specimens (12.7%) that were unrefrigerated for less than 2 h tested positive for *C. difficile*, as compared to 96 of 771 (12.5%) that were unrefrigerated for more than 2 h ($\chi^2 0.014, p=0.91$). A total of 954 specimens were unrefrigerated for less than 13 h, of these, 127 (13.3%) tested positive for *C. difficile*. One hundred specimens were unrefrigerated for more than 13 h, of those, 5 (5%) tested positive for *C. difficile* ($\chi^2 5.709, p=0.02$ [Table 1]).

A total of 193 patients were tested more than once for the presence of *C. difficile* toxins in fecal specimens using EIA (Table 2). Thirty-four patients initially tested positive for *C. difficile* (median handling time 143 min), and of those, 25 became negative (median handling time 227 min) on subsequent testing. The median time difference between testing of the initial and subsequent specimens was 7 days. Out of 159 patients who initially tested negative for *C. difficile* (median handling time 194 min), 11 became positive (median handling time 317 min) on subsequent testing. The median time difference between testing of the initial and subsequent specimens was 3 days. Out of 132 *C. difficile*-positive fecal specimens, 117 specimens were tested positive once, 12 specimens were tested positive twice, 2 specimens tested positive three times, and 1 specimen tested positive five times.

Discussion

The *C. difficile* toxins are labile [8, 11–13] and their stability can affect the result of CDI [8]. There are no large scale studies evaluating the stability of *C. difficile* toxins in human fecal specimens at room temperature. We examined how the handling of unrefrigerated fecal specimens affected the results of CDI testing using the Premier™ Toxins A and B kit.

A variety of laboratory tests are available to diagnose CDI [1]. EIA is the most widely used screening test to diagnose CDI [1, 2], since it is highly sensitive and specific [2] and has a turnaround time of 2–3 h [1, 2]. Hence, we used the EIA to diagnose CDI. Other CDI diagnostic tests with rapid turnaround time as well as high sensitivity and specificity include: glutamate dehydrogenase or common antigen test and qPCR [1]. Although tissue culture with cytotoxin production is the recognized gold standard for diagnosing CDI, it has a turnaround time of 2–3 days and therefore is not useful in clinical settings [1, 2, 16–18].

The CDC released information for healthcare providers in August 2004 (updated July 2005) stating that fecal specimens for *C. difficile* should be tested within 2 h of collection [11]. In contrast, the results of our study demonstrate that the *C. difficile* toxins can routinely be detected by EIA for more than 2 h in unrefrigerated human fecal specimens. Moreover, there was no significant difference in the yield of positive results in specimens which had a handling time of 2 h or less (12.7%) compared to those that had a handling time more than 2 h (12.5%).

![Fig. 1](image-url)
The results of our study demonstrate that CDI can be detected accurately for up to 13 h in an unrefrigerated human fecal specimen using EIA.

The disparity between the results of our study and information released by CDC is difficult to explain. We did not retest the same specimen for the presence of C. difficile toxin at different time intervals. Therefore, we could not determine the stability of the C. difficile toxins in the same specimens for more than 2 h. However, we have observed that C. difficile toxin can still be detectable for up to 13 h in a large retrospective sample of unrefrigerated fecal specimens. One possible explanation for the disparity of our findings could be related to the recent emergence of virulent strains of C. difficile. Following a CDI outbreak in Quebec, Canada in 2002 [19, 20], hospitals in that area experienced an almost 5-fold increase in incidence of CDI [2, 19, 21, 22].Typing of the strains revealed that a hypervirulent strain of C. difficile (BI/NAP1/027) was accountable for the outbreaks [2, 19]. Subsequently, the BI/NAP1/027 strain of C. difficile has been isolated throughout the United States and other countries including the United Kingdom, Belgium, Netherlands, and France [23, 24]. This strain has been found to produce 16 times more toxin A and 23 times more toxin B than the other C. difficile strains [2, 25]. The increase in production of toxins by more virulent strains could increase the amount of C. difficile toxins in fecal specimens. These higher quantities of C. difficile toxin might explain the prolonged stability and identification of C. difficile toxins in unrefrigerated human fecal specimens using EIA relative testing prior to the emergence of the virulent strain.

Our study has a number of important limitations. First, none of the positive or negative specimens were confirmed by tissue culture and cytotoxin production. Although tissue culture with cytotoxin production is the gold standard for diagnosis, EIA is sensitive and specific test for CDI and is the most widely used test in a clinical setting to diagnose CDI [1, 2]. Moreover, tissue culture only detects toxin B, requires technical expertise to perform, is expensive, and requires 24–48 h to achieve a final result [1, 11]. Second, this is a retrospective study examining how the unrefrigerated handling time of human fecal specimens affects the results of CDI testing. We did not retest the same fecal specimen prospectively at different unrefrigerated time intervals. Third, the ambient or “room” temperature in our center (20°C–25°C) may not be representative of the ambient temperature in other institutions or in environments where centralized air conditioning is not employed. An increase in ambient temperature could have an adverse impact on unrefrigerated specimen viability. Therefore our results may not be applicable in other laboratories where the ambient room temperature could be much higher. Any further studies should account for the effect of increased ambient temperature on stool sample viability. Lastly, we did not examine the impact of antibiotic treatment for CDI on detection of C. difficile toxins in fecal specimens. It is not clear how quickly patients, whose fecal specimens which are initially positive for C. difficile by EIA, become negative after initiating treatment with antibiotics.

In summary, all efforts should be made to process specimens according to the current recommendations to ensure the accuracy of EIA testing. However, if unavoidable, stool can be tested even if unrefrigerated for up to 13 h after collection. Further prospective studies are needed to evaluate the viability of C. difficile toxin in the same fecal specimen at increasing unrefrigerated handling time intervals to confirm our findings and identify the maximum unrefrigerated handling time that allows detection of C. difficile toxins by EIA.

Table 1 Effect of handling time on detection of C. difficile toxin

<table>
<thead>
<tr>
<th>Handling process</th>
<th>Total specimens (n</th>
<th>Positive specimens (n [%])</th>
<th>Negative specimens (n [%])</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1054</td>
<td>132 (12.5%)</td>
<td>922 (87.5%)</td>
<td></td>
</tr>
<tr>
<td>2 hours or less</td>
<td>283</td>
<td>36 (12.7%)</td>
<td>247 (87.3%)</td>
<td>0.917</td>
</tr>
<tr>
<td>More than 2 hours</td>
<td>771</td>
<td>96 (12.5%)</td>
<td>675 (87.5%)</td>
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</tr>
<tr>
<td>4 hours or less</td>
<td>579</td>
<td>71 (12.2%)</td>
<td>508 (87.8%)</td>
<td>0.780</td>
</tr>
<tr>
<td>More than 4 hours</td>
<td>475</td>
<td>61 (12.8%)</td>
<td>414 (87.2%)</td>
<td></td>
</tr>
<tr>
<td>8 hours or less</td>
<td>807</td>
<td>110 (13.6%)</td>
<td>697 (86.4%)</td>
<td>0.610</td>
</tr>
<tr>
<td>More than 8 hours</td>
<td>247</td>
<td>22 (8.9%)</td>
<td>225 (91.1%)</td>
<td></td>
</tr>
<tr>
<td>12 hours or less</td>
<td>924</td>
<td>120 (13%)</td>
<td>804 (87%)</td>
<td>0.259</td>
</tr>
<tr>
<td>More than 12 hours</td>
<td>130</td>
<td>12 (9.2%)</td>
<td>118 (90.8%)</td>
<td></td>
</tr>
<tr>
<td>13 hours or less</td>
<td>954</td>
<td>127 (13.3%)</td>
<td>827 (86.7%)</td>
<td>0.016</td>
</tr>
<tr>
<td>More than 13 hours</td>
<td>100</td>
<td>5 (5%)</td>
<td>95 (95%)</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated using χ² for categorical variables

Table 2 Testing frequency of C. difficile fecal specimens per patient

<table>
<thead>
<tr>
<th>Testing frequency</th>
<th>Number of times fecal specimens tested per patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of C. difficile positive fecal specimens</td>
<td>57</td>
<td>34</td>
<td>12</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Number of total fecal specimens tested</td>
<td>443</td>
<td>180</td>
<td>147</td>
<td>76</td>
<td>60</td>
<td>78</td>
<td>70</td>
<td>1054</td>
<td></td>
</tr>
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References