Comparison of Two Molecular Methods for Detecting Toxigenic Clostridium Difficile

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Abstract. Background: Clostridium difficile is one of the most common causes of nosocomial diarrhea, and diagnostic methods for detecting C. difficile infection have shifted from conventional to more recent molecular techniques. This study aimed to compare the performance of two molecular assays (Meridian Illumigene™ and AdvanSure CD real-time PCR) in detecting C. difficile using a toxigenic culture as a reference standard. Materials and Methods: This study was conducted at Kyung Hee University Hospital, a tertiary university teaching hospital in Seoul, Korea, from July 2010 to February 2011. The study used 203 fresh diarrheal stools. All fecal specimens were immediately tested by culture and the VIDAS C. difficile toxin A & B assay using an automated VIDAS immunoanalyzer. The remainder was stored at -70°C until required for AdvanSure CD real-time polymerase chain reaction and Illumigene™. The alcohol shock procedure was then performed. Aliquots were inoculated directly on C. difficile-selective agar and blood agar and then incubated in an anaerobic jar for 48 h at 35°C. The Rapid ID 32 A test was used for specifying colonies on plates. The AdvanSure CD real-time PCR was used to detect the tcdA and tcdB gene, and PCR Illumigene™ kits were used to detect the tcdA gene of the pathogenicity locus (PaLoc) harboring toxigenic C. difficile. Results: Of 203 clinical samples, 197 showed identical results between the two molecular assays, with a concordance rate of 97.0%. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were as follows: Illumigene: 92.3, 99.4, 96.0, and 98.9, respectively; AdvanSure CD real-time PCR: 84.6, 98.3, 88.0, and 97.8, respectively. Conclusions: Both molecular assays demonstrated good sensitivity and specificity. Additionally, both molecular assays showed comparable results to those of a toxigenic culture, albeit with a slight decrease in test sensitivity and specificity.

Introduction

Clostridium difficile is a common cause of nosocomial diarrhea. Known primary virulence factors of C. difficile are toxin A and toxin B, encoded respectively by the tcdA and tcdB genes. In most laboratories, the most easily accessible and frequently used method for diagnosing C. difficile-associated infection (CDI) is an enzyme immunoassay (EIA), which is simple to perform and has a short turnaround time [1,2]. However, its relatively low sensitivity has caused problems [3-5], as an accurate and timely diagnosis is necessary for both appropriate clinical management and timely implementation of infection control and pharmacy measures [6]. Toxigenic culture and cell culture cytotoxicity neutralization assays are considered the reference standard methods. However, they are labor intensive and have a comparatively long turnaround time. Diagnostic methods for detecting CDI have shifted from conventional means to molecular techniques reported to be more rapid and sensitive [7].

Many large laboratories are now replacing conventional approaches with amplified nucleic acid methods. Although recent molecular methods have advanced in cost benefit, ease of use, and turnaround time, they have not yet been widely
accepted as the sole diagnostic approach in clinical settings [8]. While these assays have shown high sensitivity and specificity compared with those of a toxigenic culture, some consider it premature for PCR assays to be used as a sole diagnostic method [9]. The Food and Drug Administration approved the Meridian Illumigene™ C. difficile assay to detect the toxin A gene (tcdA) within the pathogenicity locus (PaLoc), which uses loop-mediated isothermal amplification (LAMP) [10]. The AdvanSure CD (C. difficile) real-time PCR method (LG LifeScience, Seoul, Korea) applies real-time PCR to detect toxin A and toxin B genes.

In the present study, we sought to compare the two molecular assays for detecting C. difficile with a toxigenic culture.

Materials and Methods

Stool specimen and colony identification. Stool specimen and colony identification was conducted at Kyung Hee University Hospital, a tertiary university teaching hospital in Seoul, Korea, from July 2010 to February 2011, using 203 fresh diarrheal stools.

All fecal specimens were immediately tested by culture and the VIDAS C. difficile toxin A and B assay using an automated VIDAS immunoanalyzer (bioMérieux, Marcy l’Etoile, France). The remaining specimens were stored at -70°C until required for AdvanSure CD real-time PCR and Illumigene™ [7]. The alcohol shock procedure was performed to inhibit growth of other bacteria and improve the sensitivity of the anaerobic culture [4]. Aliquots were directly inoculated on C. difficile-selective agar (Komed, Seung-Nam, Korea) and blood agar (Hangang, Kun-po, Korea) plates, and then incubated in an anaerobic jar for 48 h at 35°C. The Rapid ID 32 A test (bioMérieux) was used for specifying colonies on plates.

Molecular assays. 1) Real-time PCR was performed using the AdvanSure CD real-time PCR Kit (LG Life Science, Daejeon, Korea). Genomic DNA was extracted directly from stool samples using a QIAamp mini stool kit (Qiagen, Germany). AdvanSure CD real-time PCR for toxin genes was carried out by first preparing DNA using a QIAamp mini stool kit (Qiagen, Hilden, Germany). AdvanSure CD real-time PCR was performed according to the manufacturer’s instructions with the SLAN real-time PCR detection system (LG Life Science, Daejeon, Korea). Plasmid DNA containing C. difficile was used as a control.

2) Illumigene™ (Meridian Bioscience, Cincinnati, OH) was used to detect tcdA of the PaLoc harboring toxigenic C. difficile. The LAMP assay was performed according to the manufacturer’s instructions. Plasmid DNA containing C. difficile was used as a control.

3) An in-house PCR test for toxin A and toxin B was performed as described previously [11]. In-house PCR was validated by control strain.

This study was approved by an appropriate Institutional Review Board.

<table>
<thead>
<tr>
<th>Result</th>
<th>Toxigenic culture</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>FPR (%)</th>
<th>FNR (%)</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
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<td>88</td>
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Illumigene™ C. difficile

<table>
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<tr>
<th>Result</th>
<th>Toxigenic culture</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>FPR (%)</th>
<th>FNR (%)</th>
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<tr>
<td>Positive</td>
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<td>176</td>
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</tbody>
</table>
Results

Concordance rate of molecular tests. Of the 203 clinical samples, 26 (12.8%) tested positive using the toxigenic culture and 197 showed identical results between the two molecular assays, a concordance rate of 97.0%. To resolve discrepancies, we used our previous in-house PCR assay results on colonies from 30 culture-positive samples. In-house PCR for toxin A and toxin B detected an additional toxin-positive sample, and AdvanSure CD real-time PCR on colonies of this sample showed a positive result. When AdvanSure CD real-time PCR was performed using cultured colonies, four specimens were positive (Table 1).

Analysis of discrepant results. Three specimens that showed negative results for AdvanSure CD real-time PCR displayed positive results for Illumigene™ and anaerobic culture, indicating that these results were false-negative by AdvanSure CD real-time PCR. Additionally, three specimens that showed positive results for AdvanSure CD real-time PCR demonstrated negative results for Illumigene™. Two of these also gave negative results for anaerobic culture and EIA assays and false-positive results for AdvanSure CD real-time PCR. One of these false-positive samples for AdvanSure CD real-time PCR showed a negative result in a repeated AdvanSure CD real-time PCR and the other was not repetitively tested because of an insufficient stool sample. The last of these three specimens showed a positive result for anaerobic culture and in-house PCR on colonies, indicating a false-negative Illumigene™ result. Of the specimens mentioned above, no positive results were obtained for EIA assays (Table 2).

To resolve discrepancies, we used our previous in-house PCR assay results [7] and this experiment results. In-house PCR for toxin A and B detected an additional four toxin-positive samples that had been negative for AdvanSure CD real-time PCR on stool samples: three were positive and one was negative for Illumigene™ on stool samples. However, the corresponding colonies turned out to be positive by these three methods.

Discussion

As C. difficile is one of the most common causes of nosocomial diarrhea, improved diagnostic algorithms based on rapid detection of toxigenic strains of C. difficile are in demand [12].

Noren reported that LAMP methods showed a sensitivity and specificity of 98%, a positive predictive value of 92%, and a negative predictive value > 99% [13]. Our results were comparable. Pancholi et al reported that Xpert C. difficile and Xpert C. difficile/Epi assays were the most sensitive ways to detect toxigenic C. difficile in the stool [14]. The Xpert CD assay is a real-time multiplex PCR assay that detects cytoxin and binary toxin genes. The advantage of the Xpert CD assay is its rapidity and simplicity [15]. The reported sensitivity and specificity of the Xpert system are 100% and 94.6%, respectively [15].

Problems such as false-negative and false-positive results should be considered when evaluating molecular methods. We reported discrepant results, particularly regarding false-negative results in a previous study [7]. False-negative cases of AdvanSure CD real-time PCR and Illumigene had positive results by AdvanSure CD real-time PCR, Illumigene, and in-house PCR from cultured colonies. The discrepant results between original stool samples and cultured colonies may come from differences in the number of bacteria or the PCR inhibitors in stools [7]. Although we did not measure the DNA concentration, the cultured colonies include more dense and larger number of bacteria compared to those from small amount of stool specimen. We therefore hypothesized that the false-negative results of both molecular tests in original stool samples were due to a low number of bacteria in original stool specimens. In addition, we previously reported that toxin A-negative and toxin B-positive results of AdvanSure CD real-time PCR assays resulted from a partially-deleted toxin A gene [7]. While the AdvanSure CD real-time PCR assay is intended to detect tcdA and tcdB genes, Illumigene™ is designed to detect the tcdA gene. Nevertheless, Kozak et al reported that Illumigene™ could
identify all known toxin A-negative and toxin B-positive (A-B+) strains that have various deletions at the 3′ end of the tcdA gene, because this assay is designed to detect the 5′ region of the tcdA gene that remains intact in all of these strains [16]. In our study, two of three specimens that had A-B+ strains resulted in Illumigene™ positive results, in agreement with Kozak et al [16]. Additionally, the remaining specimen was false-positive by AdvanSure CD real-time PCR.

In a previous study, we evaluated the selective chromogenic agar for detecting C. difficile (chromID™ CD agar, CDIF) [17]. We propose that performing the molecular assay with cultured colonies after anaerobic culture with CDIF media within 24 h can improve the detection rate of toxigenic C. difficile and reduce the turnaround time. Thus, the combination of the molecular assay and anaerobic culture can be a complementary scheme.

Luo and Banaei report a few cases that became positive when serial specimens were tested [18]. However, they insisted on repeated PCR within 7 days, which does not appear useful [18] since the amount of bacteria in a specimen may influence the results of PCR assays. A partially deleted toxin A gene was found in three specimens and resulted in ~700-bp bands after amplification by in-house PCR. These partially deleted toxin A genes were not detected by AdvanSure CD real-time PCR for the toxin A gene. Additionally, repeating PCR tests on cultured colonies might increase the sensitivity of PCR-based methods.

In conclusion, Illumigene™ and AdvanSure CD real-time PCR assays performed comparably to the toxigenic culture with a decrease in sensitivity. However, both tests showed high specificity. Thus, in the era of molecular tests, those approaches can serve as a good substitute for a toxigenic culture and EIA.

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References


