Evaluation of the rapid loop-mediated isothermal amplification assay Illumigene for diagnosis of Clostridium difficile in an outbreak situation

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An outbreak of Clostridium difficile infection (CDI) at Höglandet Hospital Eksjö in southern Sweden in 2011 was mainly due to a multidrug-resistant PCR ribotype 046 (30% of all samples). Diagnostics used routinely was the Vidas CDAB assay, but to control the outbreak the rapid loop-mediated isothermal amplification (LAMP) assay Illumigene was introduced and both techniques were compared to Toxigenic culture (TC) prospectively. The LAMP assay had a superior sensitivity, that is, 98% compared to 79% for the Vidas CDAB assay. Most importantly, the mean turn-around-time from collecting sample to result was reduced from 59 h to 2 h enabling early isolation of patients and effective hygiene precautions. This may potentially decrease the morbidity and nosocomial transmissions of C. difficile.

Key words: Clostridium difficile; diagnostics; Illumigene; LAMP; Vidas CDAB.

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INTRODUCTION

Clostridium difficile is a major cause of antibiotic associated diarrhea and an emerging threat in hospital environments (1). C. difficile infections (CDIs) have shown an increased frequency and severity over the last 10 years, mainly due to the international spread of the epidemic C. difficile NAP1/027 strain (2). CDI ranges from mild diarrhea to life-threatening pseudomembranous enterocolitis with megacolon and possible perforation (3). Patients have increased length of hospital stay, cost of medical care and mortality rate (4). Risk factors are antibiotic treatment, old age and hospitalization (5). Fast and accurate diagnostics may prevent nosocomial spread of C. difficile (6).

C. difficile produces enterotoxin A (TcdA) and cytotoxin B (TcdB), which induces cell death and inflammatory reactions in the intestine (7). These toxins can be detected for diagnostic purposes using cell culture assays (toxin B) and different enzyme immunoassays (EIAs) such as the Vidas CDAB assay (bioMerieux, Askim, Sweden). Many clinical laboratories use EIAs since these assays are rapid and easy to perform (8). However, as stand-alone tests the EIAs are not sensitive enough (9).
gold standards for CDI diagnosis are the stool cytotoxicity assay and the toxigenic culture (TC) (10). However, the stool cytotoxicity assay is not internationally standardized and quality assured, and it requires skills and facilities for cell culture, and the TC is slow and laborious. Recently, several PCR assays have been developed to increase the diagnostic sensitivity and to reduce the turn-around-time (6). However, culture will remain important since it provides isolates for molecular epidemiological typing and antimicrobial susceptibility testing (11).

At Höglandet Hospital Eksjö in southern Sweden, there has been a 10–15 times increase in the CDI incidence during the past 5 years accompanied by a high positivity testing rate well over 30%. In fact, the calculated incidence of 2.25 cases per 1000 hospital days exceeds the Ontario NAP1/027 2008 situation in Canada (12). PCR ribotyping of a limited number of C. difficile strains, however, indicated a predominant ribotype (046) that suggested an outbreak situation within the hospital. As one step to reduce CDIs in the hospital, a rapid and accurate C. difficile diagnostics regardless of time of day and weekday was established using the loop-mediated isothermal amplification (LAMP) assay (Illumigene, Meridian Bioscience; Cincinnati, OH, USA) at the local clinical chemistry laboratory in Eksjö. All specimens were analyzed directly after arrival at the laboratory, and then transported to the central microbiology laboratory in Jönköping (located 60 km from Eksjö), for routine C. difficile diagnostics, including EIA and TC. The routine methods used in Jönköping were performed once daily and only at weekdays. The rational for the local rapid testing was to optimize the patient care and to minimize the nosocomial spread by applying rigorous infectious disease control measures.

The main aim of this study was to evaluate the LAMP assay Illumigene performed at a local clinical chemistry laboratory compared to the Vidas CDAB assay and TC performed at a central clinical microbiology laboratory. Performance characteristics, including time to diagnosis from sampling, were examined. Furthermore, the isolates were investigated using PCR ribotyping and antimicrobial sensitivity testing.

**MATERIALS AND METHODS**

**Specimens**

Fecal specimens (n = 302) were collected from patients with clinical signs of possible CDI, who were admitted to Höglandet Hospital Eksjö and/or visited primary health care facilities from 21st of June 2011 to 20th of November 2011. Time to diagnosis from sampling was measured and compared to the same time period the previous year. The transportation time from Höglandet Hospital Eksjö to the central microbiology laboratory in Jönköping is approximately 90 min.

**C. difficile LAMP assay Illumigene**

Specimens were immediately subjected to C. difficile diagnostics at the local clinical chemistry laboratory, using the LAMP assay Illumigene according to the manufacturer’s instructions (Illumigene). Briefly, the fecal samples were initially filtrated and denaturated 10 min at 95 °C followed by loading of reaction tubes, each paired with a positive control. The LAMP reaction subsequently runs for 40 min presenting positive or negative results on display as well as in print.

**Culture of fecal specimens and detection of TcdA and TcdB**

Culture of C. difficile was performed anaerobically on cycloserine-cefoxitin-fructose agar according to the Swedish guidelines (http://www.referensmetodik.smii.se/w/Clostridium_difficile-laboratoriediagnostik) at the clinical microbiology laboratory (County Hospital Ryhov, Jönköping, Sweden).

Identification of TcdA and TcdB was performed on all fecal specimens using an enzyme-linked fluorescent immunoassay (ELFA, Vidas CDAB assay, bioMerieux), according to the manufacturer’s instructions.

**Toxigenic culture of C. difficile**

C. difficile isolates from toxin-negative stool specimens were examined for toxin production using the ELFA Vidas CDAB assay. Briefly, colonies (n = 2 to 3) were suspended in 2.0 ml of sterile 0.45% saline. An aliquot (200 µl) was diluted in 1 ml diluted buffer, and 300 µl was inoculated into a well. The assay was performed as described above.

**PCR ribotyping of C. difficile**

Isolates were grown anaerobically in 2 ml of peptone yeast broth for 24 h and harvested by centrifugation. DNA extraction was performed by suspending the
pellet in 0.2 ml of 10% Chelex-100 in water and boiling for 10 min prior to the PCR. PCR amplification and analysis of PCR products were performed as previously described (13). All banding patterns were analyzed by one technician blinded for epidemiological data. We used Molecular Analyst Fingerprinting Plus 1.6 software (Bio-Rad, Solna, Sweden; the software is identical with Compar of Applied Maths) and the unweighted pair group method using arithmetic averages for cluster correlation. PCR ribotypes were assigned as described elsewhere (14).

Antimicrobial susceptibility testing

This was done by using the Etest methodology (Bio-Merieux, Solna, Sweden) with bacterial suspensions according to McFarland 4 turbidometry and incubated anaerobically for 48 h on Mueller-Hinton agar supplemented with 5% sheep blood according to the instructions of the manufacturer.

RESULTS

Comparison of the LAMP assay Illumigene, Vidas CDAB and TC

The mean values of time to diagnose were 2.5 days for the old process and 2.3 h with the local Illumigene approach. This is equivalent to a mean reduction of 57 h.

The LAMP assay Illumigene detected C. difficile in 84 (28%) of the 302 specimens. Culture was successful in 98 specimens and of these 88 bacterial strains were toxin producing, resulting in 29% positive TC of all specimens (Table 2). The Vidas CDAB assay showed positive results in 61 (20%) and equivocal results in 15 (5%) specimens. Thus, CDI was indicated in 76 (25%) specimens, but confirmation was delayed significantly by culture verification and of these 15 equivocal specimens only seven toxin producing bacterial strains could be isolated. The sensitivity compared to the gold standard TC was 68% for Vidas CDAB and 95% for LAMP. The positivity rate was 37% of all requested CDI tests leading to a positive and negative predictive value of 98% and 98% for LAMP vs 98% and 90% for Vidas CDAB (Table 1).

Ribotypes of strains isolated from stool specimens

All TC positive isolates were ribotyped and belonged to 15 different PCR ribotypes (Table 2). The most dominant ribotype was 046 (30%) followed by 014 (18%) and 012 (17%). Ribotype 002 seemed to be more difficult to detect using Vidas CDAB as shown by others (15) and interestingly two non-toxigenic isolates produced equivocal result (Table 2).

Antimicrobial susceptibility

A majority of the isolates were fully susceptible to amoxicillin as well as vancomycin and metronidazole [all PCR ribotypes exhibiting minimum inhibitory concentration (MIC90) values between 0.19 and 1.5 mg/L, see Table 3]. Multidrug resistance, defined as maximum MIC (mg/L) for two or more classes of antibiotics, were found in 45% (39/88) of the toxigenic C. difficile isolates and exclusively among PCR ribotype 046 (n = 26) and 012 (n = 13). The MIC values for clindamycin, moxifloxacin, rifampicin and tetracycline were very high for these two ribotypes (Table 3).

DISCUSSION

In this study, the rapid LAMP assay Illumigene showed high sensitivity and specificity, in

<table>
<thead>
<tr>
<th>TC</th>
<th>Illumigene</th>
<th>VIDAS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.95 (84/88) CI 0.92–0.99</td>
<td>0.68 (60/88) CI 0.57–0.78</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.99 (212/214) CI 0.97–0.99</td>
<td>0.95 (205/214) CI 0.92–0.98</td>
</tr>
<tr>
<td>PPV</td>
<td>0.98 (84/86) CI 0.92–0.99</td>
<td>0.98 (60/61) CI 0.91–0.99</td>
</tr>
<tr>
<td>NPV</td>
<td>0.98 (212/216) CI 0.97–0.99</td>
<td>0.90 (205/226) CI 0.86–0.94</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; CI, Confidence interval.

1Equivocal verification by positive toxigenic culture was 7/15 and improves sensitivity to 0.76 and 0.88, respectively but delays final result by days.
Table 2. Isolated *Clostridium difficile* (n = 98) from 302 faecal CDI samples yielding 84 (28%) Illumigene positive, 61 (20%) VIDAS positive results and 88 (29%) toxin-culture positive isolates distributed according to corresponding PCR ribotype

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>No isolates</th>
<th>Illumigene assay</th>
<th>Vidas CDAB assay</th>
<th>Toxigenic culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swedish EU/UK</td>
<td></td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>SE 19c O46</td>
<td>26</td>
<td>25</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>SE 17 O12</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>SE 21b O14</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>SE 12 OO2</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>SE 16 OO5</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>SE 21 O14</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>SE 14 nd1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SE 20 OO1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other &lt;5 isolates</td>
<td>20</td>
<td>19</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Non-toxogenic Cd</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>84</td>
<td>14</td>
<td>61</td>
</tr>
</tbody>
</table>

1No corresponding EU/UK ribotype established.
2Only 7/15 of equivocal results presented toxigenic *Clostridium difficile*.

Table 3. Antimicrobial susceptibility (Etest) and PCR ribotypes among 88 toxigenic *C. difficile* isolates both Illumigene and culture positive sampled from the *Clostridium difficile* infection outbreak in Eksjö, Sweden

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>PCR ribotype (EU/UK nomenclature)</th>
<th>O46 (n = 26)</th>
<th>O12 (n = 13)</th>
<th>O14 (n = 15)</th>
<th>OO2 (n = 6)</th>
<th>Other (&lt;5 isolates, n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC90 g/L (range)</td>
<td>O46 (n = 26)</td>
<td>O12 (n = 13)</td>
<td>O14 (n = 15)</td>
<td>OO2 (n = 6)</td>
<td>Other (&lt;5 isolates, n = 28)</td>
</tr>
<tr>
<td>Anoxicilin</td>
<td>1.5 (0.5–1.5)</td>
<td>1.5 (0.5–1.5)</td>
<td>0.5 (0.5–0.75)</td>
<td>0.5 (0.5–0.75)</td>
<td>0.75 (0.125–1)</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.75 (0.5–1.5)</td>
<td>1.5 (0.5–1.5)</td>
<td>0.75 (0.38–1)</td>
<td>0.75 (0.75)</td>
<td>0.75 (0.38–4)</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.38 (0.19–0.38)</td>
<td>0.125 (0.094–0.19)</td>
<td>0.25 (0.125–0.25)</td>
<td>0.19 (0.19–0.25)</td>
<td>0.19 (0.094–0.25)</td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;256 (1.5–256)</td>
<td>&gt;256 (&gt;256)</td>
<td>3 (3–6)</td>
<td>4 (2–4)</td>
<td>2 (0.25–256)</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&gt;32 (1–32)</td>
<td>&gt;32 (&gt;32)</td>
<td>&lt;0.002 (&lt;0.002)</td>
<td>&lt;0.002 (&lt;0.002)</td>
<td>&lt;0.002 (&lt;0.002–32)</td>
<td></td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>&gt;32 (&gt;0.002–32)</td>
<td>&gt;32 (&gt;32)</td>
<td>1 (0.75–1)</td>
<td>0.75 (0.75–1)</td>
<td>0.75 (0.5–2)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>24 (0.023–32)</td>
<td>32 (24–32)</td>
<td>0.047 (0.023–0.94)</td>
<td>0.032 (0.032–0.47)</td>
<td>0.047 (0.032–12)</td>
<td></td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.047 (&lt;0.016–0.047)</td>
<td>0.047 (0.016–0.047)</td>
<td>&lt;0.016 (&lt;0.016)</td>
<td>&lt;0.016 (&lt;0.016)</td>
<td>&lt;0.016 (&lt;0.016–0.023)</td>
<td></td>
</tr>
</tbody>
</table>

1One single 017 isolate represent multiple resistance i.e., MIC clindamycine >256, rifampicin >32, moxifloxacin >32 and tetracycline 12 mg/L.

accordance with other recent studies (16–18). Furthermore, we detected a high prevalence (29%) of *C. difficile* in collected specimens and PCR ribotyping discovered 046 (19c in the Swedish nomenclature) as the dominating ribotype. Ribotype 046 (as well as the identified ribotype 012) was multidrug-resistant and accordingly, prone to nosocomial selection and subsequent risk for silent outbreak. Similar prevalence and level of antimicrobial resistance in this ribotype have also been previously described (19). The marked decrease in time to diagnosis using the rapid LAMP assay Illumigene makes early infectious disease control measures possible, probably resulting in less nosocomial transmission (6). In addition, some patients may receive relevant therapy faster with decreased CDI morbidity as a possible result.

The LAMP assay Illumigene is innovative, and the target (tdcA) differs from many real-time PCR-based methods, which are based on the *tcdB* gene (20). *C. difficile* strains have variable toxin A and B genes (21), but the targeted region of tdcA appears to be highly conserved independent on toxin A production, minimizing the risk for false negative results due to sequence alterations.

Recent evaluations of some commercially available diagnostic real-time PCR-based showed sensitivity and specificity ranging from 77.3 to 97.1% and 93 to 100%, respectively, compared to gold standard (8). In this study, the LAMP assay Illumigene had a sensitivity and specificity of 95% and 99%, respectively, which is concordant with previously published data for the LAMP assay Illumigene (18). The
performance characteristics of the LAMP assay Illumigene makes it suitable as a rapid, effective and reliable stand-alone diagnostic test, for diagnostics near to the patient as well as in various laboratory settings. The advantage of a benchdesigned closed nucleic acid amplification test that is free from critical PCR-laboratory specifications is obvious and at present 3 years without contamination problems or mismatch compared to toxigenic culture makes it quite safe to use.

The Vidas CDAB assay displayed suboptimal sensitivity, and should not be recommended as a single test for diagnostics, which also has been stated by others (9). Toxigenic culture, although time-consuming, remains a very sensitive diagnostic technique. In the present, only two discrepant specimens became positive in the LAMP assay but negative in culture. This could be due to a superior sensitivity of the LAMP assay but further verification will be done. On the other hand, four isolates grew positive from a negative LAMP specimen and could instead reflect a lower sensitivity in the LAMP assay (Table 1 and 2).

In summary, we show that the LAMP test is rapid, easy to perform, and accurate for diagnosing CDI, also in settings without a clinical microbiology laboratory. It may potentially decrease the morbidity and nosocomial transmissions of C. difficile. Rapid results may have contributed to control of the outbreak at the hospital in Eksjö, Sweden.

We thank Josefin Andersson for technical assistance, isolating and ribotyping C. difficile isolates. We also thank Karsten Offenbartl for the initiative to establish local diagnostics in Eksjö and Michael Toepfer for providing epidemiological data.

REFERENCES


