An evaluation of laboratory methods for the diagnosis of toxigenic Clostridium difficile infection: enzyme immunoassays for glutamate dehydrogenase and toxins A and B, real time PCR (tcdC gene) and stool culture

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Abstract

Background: Clostridium difficile is a major cause of health-care associated infections (HAI) and is the principle cause of diarrhoea in hospitalised patients. Enzyme immunoassays (EIAs) that detect C. difficile toxins A and B are commonly used by the laboratory for the diagnosis of toxigenic C. difficile infection (CDI). However, unacceptably low sensitivities and specificities for these toxin EIAs have been reported and attention has been directed at investigating more sensitive assays such as PCR and the use of algorithms for the detection of toxigenic C. difficile in order to improve the laboratory diagnosis.

Objective: To evaluate six tests for the laboratory diagnosis of C. difficile disease individually and in combination in terms of their sensitivity, specificity, negative predictive value, positive predictive value, timeliness and economy.

Methods: Diarrhoeal stool samples (n=346) were tested by a glutamate dehydrogenase, three EIAs for C. difficile toxins A and B, a real time (RT)-PCR to detect the tcdC gene and culture. The results of these assays were compared with a reference stool culture method using C. difficile agar followed by a toxin EIA on broth subcultures and the LightMix C. difficile RT-PCR for the tcdC gene on isolates subcultured on fastidious anaerobe agar. The performances of the individual assays and in combination were assessed and the results compared to reference culture.

Results: Fifteen specimens (4.3%) were considered positive for toxigenic C. difficile when compared to the reference standard. The sensitivities, specificities, positive predictive values (PPVs) and negative predictive values (NPVs) respectively were 86.7%, 96.7%, 54.2% and 99.4% for the Teclab C.DIFF Chek 60, 80%, 100%, 100% and 99% for the Teclab C. Diff Tox A/B, 80%, 90.2%, 66.7% and 99.1% for the Meridian Premier Toxin A/B, 93.3%, 99.7%, 92.3% and 99.8% for the Immunocard A/B and 93.3%, 99.7%, 93.3% and 99.7% for the LightMix C. Difficile RT-PCR.

Conclusions: The C. difficile toxin A/B EIA kits failed to detect 3 of 15 true positive samples. The GDH EIA was not sensitive enough to be used as a screening assay in combination with any toxin A/B EIA. Combining the GDH EIA with any other test would increase workload, cost and turnaround time with no improvement in sensitivity. The best performing individual test in regards to sensitivity and turnaround time was the LightMix C. difficile tcdC RT-PCR. The best performing combination of assays was both a toxin A/B EIA on stool and culture. This would allow the reporting of 97.7% of results within 48 hours with 100% sensitivity and specificity.

Key words: Clostridium difficile, glutamate dehydrogenase, toxins A and B, real-time PCR, culture, algorithm, sensitivity, specificity, positive predictive value, negative predictive value.


Introduction

Clostridium difficile is a major cause of health-care associated infections and is the principle cause of diarrhoea in hospitalised patients, particularly in those with a recent history of antibiotic use (1). The organism is a spore-forming gram-positive anaerobic bacillus and pathogenesis is related to the production of toxin A, an enterotoxin and toxin B, a potent cytotoxin (2,3). C. difficile can cause a range of clinical symptoms from mild to severe diarrhoea and more seriously, pseudomembranous colitis, toxic megacolon, bowel perforation, sepsis and death (4).

The incidence of C. difficile infection has been increasing in individuals considered at-risk; i.e. individuals in health care facilities, those with a recent history of hospitalisation, prior use of antibiotics or chemotherapeutics, the elderly and immunocompromised (1,5). There are also reports of an increased incidence of C. difficile infection in populations previously considered to be low-risk, including children, pregnant women and previously healthy individuals with no known risk factors (4,6). Certain strains of C. difficile have also been associated with increased severity and mortality (7,8).

The economic burden of C. difficile infection is significant and in the United States it has been estimated that the overall annual cost of management of C. difficile infection exceeds $1.1 billion dollars (9). The considerable costs associated with the management of C. difficile infection as well as the apparent increased incidence and severity of C. difficile infection has led to renewed interest in the laboratory diagnosis of the disease.

Laboratory tests available for the diagnosis of C. difficile infection include the cell cytotoxicity neutralisation assay (CCNA), culture, enzyme immunoassays (EIAs) and PCR. The majority of laboratories use EIAs to detect C. difficile toxins A and B as they are reasonably rapid and inexpensive (10,11). However, the value of toxin EIAs for diagnosis has been debated over the past few years due to reported low sensitivities and poor positive predictive values (12-17). This has led many to look at alternative methods to improve the laboratory diagnosis of C. difficile infection including highly sensitive PCR assays that target the toxin genes (mainly tcdB and tcdC) and the use of algorithms that utilise two or more diagnostic techniques (14,18,19).

The introduction of new, possibly more virulent C. difficile strains to New Zealand requires improved laboratory methods for C. difficile infection diagnosis. Incorrect laboratory results due to either lack of sensitivity or specificity can lead to inappropriate patient care with an increased possibility of cross-infections, subsequently increasing the financial burden on the health care system.
Materials and methods

Study description

Consecutive stool specimens were collected from March 2010 through to August 2010 from patients aged >20 years being investigated for the cause of their diarrhoea. Specimens were excluded from the study if another pathogen was identified. All stool samples included in this study were unformed and contained a minimum of 2.0 mL of sample to enable all testing to be performed. All specimens included were kept at 2-4°C and were less than 48 hours old when they arrived at the laboratory. Specimens were divided into multiple vials and frozen at -70°C until testing. Specimens had all assays performed within 6 weeks of storage and were thawed only once before testing. There were two duplicate patient specimens and these were collected at least 13 days apart. Ethical approval was obtained for this project from the New Zealand Health and Disability Ethics Committee - Upper South A Region.

Assays

All stools included in the study were tested for glutamate dehydrogenase (Techlab C.DIFF CHEK-60), C. difficile toxins A and B (Techlab CDIFF TOX A/B II, Meridian ImmunoCard Toxins A/B, Meridian Premier Toxins A & B), and a real-time PCR assay that targets the tcdC gene (LightMix Kit C. difficile, TibMolBiol, distributed by Roche Diagnostics NZ) and by culture. All commercial EIAs and the RT-PCR were performed according to the manufacturer’s instructions.

For the RT-PCR, a 1:3 dilution of the stool was made in STAR Buffer (Roche Diagnostics), thoroughly mixed by vortexing and then centrifuged for 1 minute at 3500rpm (550 x g). The supernatant was removed and frozen at -70°C until testing. Extraction for PCR was performed on 200 µL of supernatant by the MagNA Pure LC (Roche Diagnostics). For the PCR on cultured isolates, 3-5 colonies from fastidious anaerobe agar (FAA) subculture were placed into a vial containing 200µL PCR grade water, 180µL of MagNA Pure Bacterial Lysis Buffer (Roche Diagnostics) and 20µL of Proteinase K (Roche Diagnostics). Samples were vortexed and frozen at -70°C until testing and processed on the MagNA Pure LC as previously described. All extracted samples were run on the LightCycler 2.0 (Roche Diagnostics) in 20µL reactions for real-time amplification and detection. A non-template control (PCR grade water) and two positive controls that were provided with the kit (wild-type and 18bp del. mutant) were included with each batch.

Culture

Thawed stools were mixed with 95% ethanol in a 1:1 ratio and incubated at room temperature for 30 minutes (20). Four drops (200µl) of diluted stool was plated onto C. difficile selective media (CDA, Fort Richard Laboratories Ltd, NZ). This media is supplemented with 8 mg/L cefoxitin, 250 mg/L D-cycloserine and 5% defibrinated horse blood. Plates were incubated in anaerobic jars using anaerobic pouches (Anaero-Pack™, Mitsubishi Gas Chemical Company Inc. distributed by Ngaio Diagnostics) at 35°C for 48 hours. After 48 hours incubation, isolates resembling C. difficile, based on typical morphology and characteristic odour, were subcultured onto fastidious anaerobe agar (FAA, Fort Richard Laboratories Ltd, NZ) anaerobically for a further 48 hours at 35°C. Colonies were then checked for fluorescence under U.V. light and were gram stained. Isolates from FAA (three to five colonies) were sampled and stored for PCR and three to five colonies were subcultured in Brain-Heart Infusion broth (BHI, Fort Richard Laboratories Ltd, NZ) for 48 hours at 35°C anaerobically to allow for toxin production. The BHI broth supernatant was then tested for toxins A/B by the ImmunoCard Toxin A/B as per stool protocol. (NOTE: kit only validated by manufacturer for use on stools).

Table 1. Performance characteristics of four EIAs, a RT-PCR and culture used to detect C. difficile and C. difficile toxins A and B

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. DIFF CHEK-60 GDH (Techlab)</td>
<td>86.7</td>
<td>96.7</td>
<td>54.2</td>
<td>99.4</td>
</tr>
<tr>
<td>C. DIFF TOX A/B (Techlab)</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>Premier Tox A/B (Meridian)</td>
<td>80</td>
<td>98.2</td>
<td>66.7</td>
<td>99.1</td>
</tr>
<tr>
<td>ImmunoCard Tox A/B (Meridian)</td>
<td>80</td>
<td>99.7</td>
<td>92.3</td>
<td>99.1</td>
</tr>
<tr>
<td>LightMix tcdC PCR (TibMolBiol)</td>
<td>93.3</td>
<td>99.7</td>
<td>93.3</td>
<td>99.7</td>
</tr>
<tr>
<td>Culture†</td>
<td>93.3</td>
<td>98.8</td>
<td>77.8</td>
<td>99.7</td>
</tr>
</tbody>
</table>

* Results of 346 stools compared with the reference culture technique described in methods. † Stools ethanol shocked prior to culture on CDA followed by subculture onto FAA. PPV = positive predictive value, NPV = negative predictive value.

Results

C. difficile was detected in 19 (5.5%) of 346 stools (Table 1). Fifteen (79%) were toxin producing strains and four (21%) were non-toxin producing strains. Overall, 4.3% of specimens were considered as true positives for toxigenic C. difficile when compared to the reference standard. The sensitivities, specificities, PPVs and NPVs are shown in Table 1.

Of the 15 toxigenic isolates, 10 stools tested positive by all assays, one stool tested positive by all assays except culture and this was considered as a true positive for the purpose of calculations. On repeat culture, C. difficile was isolated from this specimen. Two stools tested positive by culture and PCR and negative by all other assays. One stool tested positive by culture, PCR and GDH only. One stool tested positive by all assays except PCR.

One sample was positive by the PCR assay alone and this was considered a false positive as C. difficile was not isolated by culture. There was one false positive toxin result by the ImmunoCard Tox A/B and six false positives for the Meridian Premier Tox A/B kit. There were 16 initially invalid ImmunoCard A/B assays due to blockage of the membrane. These were negative when a new dilution was made and centrifuged in 1.5mL eppendorf tubes for 10 seconds (at 550 x g) prior to testing. Although not specifically recommended by the manufacturer to avoid blockages, this was done to obtain a valid result. Eleven positive GDH results were considered as false positives; culture was negative for eight of these and non-toxigenic C. difficile isolates were cultured from three. There were three false negative GDH results (one non-toxigenic isolate and two toxigenic isolates were cultured). All true positive samples that tested positive by toxin A/B EIA also tested positive by the GDH assay. The performance of the two and three step algorithms are compared in Table 2.
Table 2. Performance of two-step and three-step algorithms for detecting toxin-producing *C. difficile* compared to reference culture

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH + Techlab Tox A/B</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>GDH + Prem. Tox A/B</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>GDH + I/Card A/B</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>GDH + PCR</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>GDH + Toxigenic Culture</td>
<td>86.7</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
</tr>
<tr>
<td>GDH + Techlab Tox A/B + PCR</td>
<td>86.7</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
</tr>
<tr>
<td>Culture + ImmunoCard Tox A/B on isolates</td>
<td>93.3</td>
<td>100</td>
<td>100</td>
<td>99.7</td>
</tr>
<tr>
<td>Culture + PCR on isolates*</td>
<td>93.3</td>
<td>100</td>
<td>100</td>
<td>99.7</td>
</tr>
<tr>
<td>Techlab Toxin A/B (stool) and Culture (+/- toxin testing of isolate)*</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* One toxigenic *C. difficile* isolate was repeatedly inhibited in PCR and was excluded from the calculation. † Performing both stool toxin EIA and culture as routine on all stools and performing a toxin assay on the isolate only if stool toxin is negative.

Discussion

The measured sensitivities of the *C. difficile* toxin A/B EIAs and the GDH EIA in our study were similar to those reported by Eastwood *et al.* (16) when compared to toxigenic culture (Table 1). All three toxin A/B EIAs failed to detect toxin in three of 15 true positive stools (the same specimens) and of these, GDH was not detected by EIA in two. These two samples were positive by PCR and culture only, suggesting that these may have been early infections where the better sensitivity of culture and PCR were able to detect *C. difficile* sooner than the less sensitive EIAs. One patient had an additional stool submitted two weeks later and all assays were positive at this time. The LightMix tcdC PCR and the culture method used were the best performing single tests, although the PCR assay has a much faster turnaround time with results available within hours. In the case of culture, negative cultures can be reported within 48 hours, while positive cultures could take up to 6 days to confirm.

The PPVs of the C.DIFF TOX A/B, the ImmunoCard A/B and the LightMix PCR in our study were higher than expected, despite a low number (4.4%) of toxigenic *C. difficile* (Table 1). The PPV of the Premier Tox A/B kit was lower than the other two toxin EIAs (66.7%) due to the six false positive results, results similar to other reports (21).

There have been contradictory reports on whether the GDH assay is sensitive enough to be used as a screening assay. The Techlab C. DIFF CHEK-60 GDH EIA used in our study has been shown in various reports to be an excellent screening assay with sensitivities approaching 100% and high NPVs (14, 17, 22-24), while other reports have found significantly lower sensitivities (16, 25). The sensitivity of the GDH assay in our study was only 86.7%, similar to those reported by Eastwood *et al.* (16) and Larson *et al.* (25), suggesting that it is not suitable as a screening assay, missing approximately 15% of CDI cases.

The LightMix *C. difficile* PCR detects both the wild-type and *tcdC* 18bp deletion. No strains with an 18bp deletion were detected in our study. We detected 14 of 15 true toxigenic *C. difficile* cases on stool with one false positive result giving a sensitivity of 92.3% and a specificity of 99.7% (Table 2), similar to other reports of commercially available molecular methods (16, 19, 25). Our results were slightly better than those obtained by Sloan *et al.* (15) using a PCR that targets the *tcdC* gene (wild type and the 18bp deletion mutant) and performed on the Roche LightCycler™. They found a sensitivity of 86% and a specificity of 97% compared to a reference standard of culture and PCR to detect the toxin genes. Six culture positive specimens were negative by PCR with the authors suggesting that the efficiency of nucleic acid extraction or sampling may have been the cause (15). As the false negative PCR in our study was re-extracted and repeated by PCR and remained negative (internal control returned a positive result), this was unlikely to be the cause of the PCR failure. It is possible that certain substances in stool samples may interfere with the PCR reaction but as there was no inhibition of the reaction, it is unclear why the PCR did not detect the organism. It may also be possible that this strain has a deletion in the target gene, *tcdC* interfering with primer binding. When the cultured *C. difficile* isolate from this same patient was tested by PCR it was inhibited (the internal control gave a negative result). The sample was re-extracted and the PCR repeated as before and also in a 1:3 dilution but it remained inhibited. All four EIAs performed on this stool sample were positive and broth culture tested positive by toxin A/B EIA.

There was one false negative culture in our study and when culture was repeated, toxigenic *C. difficile* was isolated. All other assays were positive; therefore the sample was considered a true positive despite the initial failure of culture. It is unknown why culture failed but, as this specimen was noted to be particularly mucoid, perhaps the organism was unevenly distributed in the stool.

As the specificity of the GDH assay is low, this assay must be used in combination with a toxin detection assay. In our study there was no advantage in the use of a two-step algorithm using the C.DIFF CHEK-60 (GDH) EIA as a screen followed by a toxin A/B EIA as a confirmatory assay as the sensitivity of the GDH assay was only slightly better than that of the toxin EIAs. If a GDH assay was used as a screening assay it would incur a significant cost to the laboratory as well as increase the workload, although it would have improved the PPV of the Premier Tox A/B EIA due to the six false positive samples found with this assay. Twelve samples that were tested by both the GDH and the toxin A/B assays were GDH positive but toxin A/B negative. These samples would require additional testing due to the low sensitivity of the toxin assays. If PCR was the third step, only one additional sample would have been determined as being a true positive. Twelve others would have been classified as
true positives as they were both GDH and toxin positive and the remainder were GDH negative. More than 24 additional tests would have been performed on this group of 346 samples. An algorithm including GDH as a screen could have been useful if there were a lot of false positive toxin A/B EIA results and poor PPVs, as seen in other reports (29). This was not seen in the population tested here, despite a low number (4.3%) of toxin-producing C. difficile. Novak-Weekley et al. (19), using toxicigenic culture as a gold standard, also found no improvement in sensitivity using a two-step algorithm (GDH and toxin A/B EIA) over the toxin A/B EIA alone. They found a sensitivity of only 55.6% for the two-step and a sensitivity of 58.3% for the individual toxin assay. There was an improvement in sensitivity to 83.1% when CCNA was included as a third step. In contrast, others have found significant improvement in results for similar two-step algorithms, i.e., a GDH assay followed by a toxin A/B EIA over the use of a toxin assay alone (17).

A possible limitation of our study was the low sample size and low prevalence of toxicigenic C. difficile. The majority of patients included in our study were considered low-risk for CDI (only 70 of 346 had C. difficile testing specifically requested). The remaining 275 samples were mainly from community patients being investigated for the cause of their diarrhoea and no toxicigenic C. difficile was found in this group. Three of the four non-toxicigenic strains found in this study were harboured by ‘low-risk’ patients and one was isolated from a patient suspected of having CDI.

Individual laboratories need to determine what test or algorithm is both practical and feasible for them to perform. An ideal algorithm or test would need to balance turnaround time, cost, sensitivity and specificity, NPVs and PPVs against available resources and it is likely that different algorithms may be necessary depending on requestor testing patterns and the prevalence of CDI in the population being tested (27). Real-time PCR was the best performing single assay overall, particularly in regards to turnaround time and sensitivity, enabling clinical decisions to be made rapidly. In laboratories with adequate resources, it is a feasible alternative to using toxin and GDH EIAs for the detection of toxicigenic C. difficile.

Not all laboratories have the required resources such as money, time and facilities to be able to perform specialised testing such as PCR. The increased cost of PCR over that of a toxin EIA (>$20.00 vs. ~$7.00) may make PCR an unlikely option for a routine diagnostic assay for CDI. It is possible that specimens could be sent to a reference laboratory for testing, but this too poses problems as turnaround times are affected.

Culture of C. difficile onto selective media following ethanol or heat shock of the stool would allow negative results to be issued within 48 hours, as culture is highly sensitive. In our study, ethanol shocking of the stool prior to plating on CDA selective agar improved the isolation rate compared with no pre-treatment of the stool and culture onto pre-reduced CDA selective agar (results not shown). Performing a highly sensitive and specific assay such as a PCR directly on the colonies to detect the toxin genes is a possibility and if performed the same day, would allow reporting of the positive cultures within 48 hours. The cost of culture is much less than PCR and toxin A/B EIAs and only culture positive samples would need further testing to determine whether the isolate is toxin producing.

Another possible option is to perform both a stool toxin A/B EIA and culture on all samples and interpreting the results of the two assays in combination. This combination would have detected all cases of CDI in our study. This option may be a feasible alternative and perform better overall than a multi-step algorithm. Some confirmatory testing may be required but this would be limited, particularly in a low prevalence setting. Delmée et al. have recommended simultaneously performing both culture and toxin A/B assay on stools (28). Samples with a positive culture but a negative toxin assay on stool would then have a toxin assay such as PCR performed on the isolate. If a combined testing protocol was used on the set of samples tested in our study, 100% of cases would have been detected and 97.7% of results could have been reported within 48 hours (100% specificity and sensitivity). Few specimens would require further testing such as PCR or EIA on the isolate, keeping costs reasonably low for the majority.

Many practitioners start empirical treatment for C. difficile infection before any laboratory results are issued and, ultimately, the diagnosis of C. difficile infection by the physician takes into account relevant clinical data in combination with laboratory results. However, false negative laboratory results due to the poor sensitivity of commercially available C. difficile toxin A/B and GDH EIAs could possibly increase the risk of cross-infections in health care facilities if these results are interpreted in isolation. Cross-infections will subsequently increase the financial burden on the health care system.

Introducing either a highly sensitive RT-PCR such as the LightMix Kit used in our study or the two-assay combination of toxin EIA and culture would increase the number of diagnosed cases of CDI and may lead to improved clinical outcomes due to appropriate patient management. The workload and costs will inevitably increase in laboratories that have historically used toxin A/B EIAs for C. difficile infection diagnosis, but if more reliable results are being reported, cost savings will be made elsewhere in the health system.

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Author contributions
Angela Horridge performed all the analytical work and data analysis and wrote the article. Mary Nulsen and Rosemary Ikram contributed to the planning and execution of the study and the substantial drafting of the article. The authors declare no conflicts of interest.

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