

# Laboratory diagnosis of *Clostridium difficile* infection. An evaluation of tests for faecal toxin, glutamate dehydrogenase, lactoferrin and toxigenic culture in the diagnostic laboratory

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## Introduction

*Clostridium difficile* was first recovered from the faeces of neonates by Hall and O'Toole in 1935.<sup>1</sup> This discovery of a new organism, then named *Bacillus difficilis*, did not, at the time, give any clue as to its future medical importance as a cause of antibiotic-associated diarrhoea and colitis. In 1977 Bartlett in America<sup>2</sup> and Larson in the UK<sup>3</sup> demonstrated that an antibiotic-induced colitis in hamsters and a cytotoxin present in the faeces of patients with pseudomembranous colitis both occurred as a result of intestinal infection with *C. difficile*.

The numbers of *C. difficile* infections in England, Wales and Northern Ireland have shown an increase from 2500 cases in 1990 to approximately 65,000 cases in 2007. This increase reflects a true rise in incidence of the disease as well as improved laboratory testing and reporting.

The majority of cases of *C. difficile* infection (CDI) are said to occur in patients over the age of 65 years, and mandatory reporting of disease in this group is now a Department of Health requirement. The accuracy of the figures, however, may be in dispute as some current laboratory methods used for diagnosis are insensitive<sup>4</sup> and approximately 20% of patients suffer recurrence of symptoms. It is not known how many of these recurrences are a relapse of the original infection or are due to the acquisition of a new strain. These new acquisitions could be classed as new infections, which would add to the total figures. Diagnosis early in the process of infection is likely to reduce the number of outbreaks.

Recurrence of infection is said to be more likely in patients who fail to make a good antibody response to the *C. difficile* toxins following infection, as patients with a good antibody response tend not to suffer repeated infections.<sup>5</sup>

Infection with *C. difficile* starts with the ingestion of spores by a patient whose normal intestinal flora has been disrupted by broad-spectrum antibiotics or other drugs that have antimicrobial activity. The spores are resistant to acid

## ABSTRACT

Faecal samples from 1007 patients suspected of having diarrhoea caused by *Clostridium difficile* infection are investigated for the presence of toxins A and B and for the presence of *C. difficile*-specific glutamate dehydrogenase (GDH). Toxigenic culture is performed on all samples and is used as the 'gold standard' for the purpose of the study. A marker for intestinal inflammation, faecal lactoferrin, is used on any samples that give a positive result in any of the above tests. Part of the study also involves an assessment of six commercial toxin kits to detect the presence of *C. difficile* toxins in faecal samples. This study revealed that the commercial toxin detection kits used can give rise to false-positive and false-negative results and that all demonstrated poor sensitivity when compared to the gold standard of toxigenic culture. Testing of faecal samples for GDH can be useful as a negative screening method as the results of this test show high correlation with culture. Faecal toxin testing can then be performed on all GDH-positive samples (GDH positivity is independent of toxigenicity in strains of *C. difficile*). The combined use of GDH and toxin testing, coupled with toxigenic culture, revealed that some patients with diarrhoea who harboured toxigenic strains of *C. difficile* were faecal toxin-negative. Lactoferrin appears to be a useful marker for the presence of inflammatory diarrhoea.

KEY WORDS: Antibiotic-associated diarrhea.  
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Colitis.  
Glutamate dehydrogenase.  
Lactoferrin.  
Toxigenic culture.

and they pass through the stomach into the small intestine where they germinate. This germination process is aided by bile salts. The vegetative cells then establish themselves in the disrupted colon where they produce two high-molecular-weight protein toxins, toxin A (an enterotoxin) and toxin B (a cytotoxin).<sup>6</sup>

The human colon is highly sensitive to these toxins, which together give rise to a profuse inflammatory diarrhoea secondary to destruction of the lining of the colon. Clinical disease may range from a mild, self-limiting diarrhoea to severe manifestations such as colitis, pseudomembranous colitis and toxic megacolon.<sup>7</sup> *C. difficile* is associated with

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95–100% of cases of pseudomembranous colitis, 60–75% of cases of antibiotic-associated colitis and 35% of cases of antibiotic-associated diarrhoea cases. The other causes of antibiotic-associated diarrhoea are largely unknown.

Hospitalised adults may become asymptomatic carriers after receiving antibiotics. Indeed, the proportion of patients who become carriers during their stay in hospital may exceed the proportion of patients with true *C. difficile* infection.<sup>8</sup>

Elderly asymptomatic patients represent a potential problem as many of these patients will at some time be cared for in long-term care facilities where *C. difficile* may become endemic.<sup>9</sup> Such carriers may harbour large numbers of *C. difficile* in their faeces. It also appears that asymptomatic patients tend to have lower levels of faecal toxin, even when high numbers of the organism are present in their faeces.<sup>10</sup> Non-toxigenic isolates do not produce diarrhoea.

Thus, clinicians and microbiologists should consider the laboratory results in conjunction with the clinical history because not everyone with *C. difficile* or its toxins become unwell.<sup>10</sup> Furthermore, diagnostic difficulty may arise in patients who are colonised with toxigenic strains but whose diarrhoea is unrelated to *C. difficile* (such as those on nasogastric feeding).

The aim of the present study is to compare various commercially available toxin kits for their ability to detect *C. difficile* toxins in the faeces of patients with diarrhoea. Other laboratory tests that may be of help to the clinician in supporting a diagnosis of CDI are also evaluated.

## Materials and methods

One thousand and seven specimens of liquid faeces from patients suspected of having CDI were examined in this study. The first 500 samples were examined for the presence of *C. difficile* toxins using six different commercially available toxin testing kits (designated TK1 to TK6). All faeces were also tested using a commercially available kit for the detection of *C. difficile*-specific glutamate dehydrogenase (GDH). All kits were used according to the individual manufacturer's instructions and only liquid faeces taking the shape of the container were tested. Faeces more than 24 hours old were excluded from the study.

All samples were cultured using an alcohol shock method,<sup>11</sup> and three drops of the faecal layer were cultured on Brazier's CCEY medium (Oxoid, Basingstoke, UK) and incubated for 48 h anaerobically. Colonies suspected of being *C. difficile* were confirmed by subjecting a 48 h pure culture on blood agar to ultraviolet (UV) light and examining for yellow-green fluorescence. Colonies were further tested using a *C. difficile* latex agglutination test (Oxoid). The odour of the culture was also noted and colonies were examined for indole production using spot indole reagent (Bioconnections, Wetherby, UK).

Confirmation of an isolate as *C. difficile* was determined using the results as listed in Table 1.

All *C. difficile* isolates were then tested for toxigenicity by picking five colonies from the 48-h blood agar culture into toxin kit diluent and testing for toxin using the Quik-Chek AB kit (Techlab, Blacksburg, USA). Strains negative by this method were subsequently inoculated into 3 mL nutrient broth, incubated anaerobically for 48 h and then tested for toxins using the Quik-Chek AB kit (Techlab). This method is the so-called 'toxigenic culture' method.

All faecal samples that tested positive in any one or more of the above commercial tests, or that were positive by the toxigenic culture method, were investigated for the presence of the intestinal inflammatory marker lactoferrin using the IBD Eze-Vue kit (Techlab).

Toxin testing (using our routine test kit; one of those tested in the first half of the study), toxigenic culture, the detection of GDH and the detection of faecal lactoferrin were performed on a further 507 liquid faecal samples as a battery of tests to investigate their usefulness in achieving a rapid and clinically useful diagnosis of CDI. Therefore, all 1007 samples were tested for toxins A and B, GDH and underwent toxigenic culture. Additionally, faeces that tested positive in any one of these tests were then tested for the presence of lactoferrin.

## Results

A total of 1007 liquid faeces were examined in this study. Of these, 123 were positive for GDH, 35 were positive for *C. difficile* toxins, 83 grew a toxigenic isolate on culture, 32 grew non-toxigenic isolates and eight showed no growth on Brazier's medium.

Eight hundred and eighty-four faecal samples were GDH- and faecal toxin-negative. Of these, four grew toxigenic isolates and two grew non-toxigenic isolates. These six samples with a 'false-negative' GDH were thought to reflect colonisation with *C. difficile* (all six were negative for lactoferrin).

Isolates were confirmed as *C. difficile* using fluorescence under long-wave UV light, agglutination with *C. difficile* latex reagent and the presence of indole, as described previously (Table 1).

The first 500 faecal samples were tested using six different commercial toxin kits (three enzyme-linked immunosorbent assay [ELISA] kits and three flow-through devices), as well as screening for GDH. The toxin detection kits were designated TK1 to TK6. The results obtained with these kits are given in Table 2. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) are given in Table 2. Sensitivity of these kits differs markedly when toxigenic culture is used as the gold standard

**Table 1.** Identification of isolates on Brazier's medium.

|                 | <i>C. difficile</i> | <i>C. innocuum</i> | <i>C. glycolicum</i> | <i>C. sordellii</i> |
|-----------------|---------------------|--------------------|----------------------|---------------------|
| Odour           | 'Elephant house'    | Burnt wood         | Sweet, sickly        | Pungent             |
| CD latex        | +                   | –                  | +                    | –                   |
| UV fluorescence | Yellow-green        | Yellow-green       | –                    | –                   |
| Spot indole     | –                   | –                  | –                    | +                   |

**Table 2.** Commercial test kit performances and toxigenic culture (first 500 samples).

|                 | GDH         | TK1 (card)  | TK2 (card)  | TK3 (ELISA) | TK4 (ELISA) | TK5 (ELISA) | TK6 (card)  | Culture-positive (toxigenic culture-positive) |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|---|
| Positives       | 54          | 18          | 21          | 19          | 18          | 18          | 18          | 57 (47)                                       |
| False positives | 2           | 0           | 2           | 0           | 1           | 2           | 0           |   |
| False negatives | 3           | 3           | 0           | 2           | 2           | 3           | 2           |   |
| Sensitivity     | 94.7 (100)* | 85.7 (31.6) | 100 (44.7)  | 90.5 (40.4) | 90.0 (31.6) | 85.7 (38.3) | 90.0 (38.3) |   |
| Specificity     | 99.5 (98.5) | 100 (100)   | 99.6 (99.6) | 100 (100)   | 99.8 (99.3) | 99.6 (99.6) | 100 (100)   |   |
| PPV             | 96.4 (87)   | 100 (100)   | 91.3 (91.3) | 100 (100)   | 94.7 (85.7) | 90 (90)     | 100 (100)   |   |
| NPV             | 99.3 (100)  | 99.4 (93.9) | 100 (94.5)  | 99.6 (94.2) | 99.6 (93.9) | 99.4 (93.9) | 99.6 (94)   |   |

\*Values cf. other toxin tests (cf. 'toxigenic culture')

compared to the use of at least two kits showing positivity as the standard. All kits demonstrated some false-positive and/or false-negative results.

Testing for GDH has utility as a 'negative screen' because it parallels culture closely and has an NPV of 99.3–99.5% (Table 3). However, GDH does not solely predict the presence of toxigenic strains and thereby necessitates the test for faecal toxin on all GDH-positive faeces.

Testing for faecal lactoferrin was used on all faeces that were positive by any test (faecal toxin, GDH or toxigenic culture).

Of the 35 faeces positive for toxin, 28 were positive for lactoferrin, indicating the presence of intestinal inflammation (colitis). The other seven toxin-positive faeces samples were lactoferrin-negative, suggesting milder diarrhoea.

Of interest were the 48 patients whose faeces were toxin-negative but who grew a toxigenic isolate on culture. Eighteen of these were lactoferrin-positive, suggesting that these patients had inflammatory diarrhoea with *C. difficile* but without the presence of detectable levels of toxin in the faeces. Examination of the laboratory records for these 48 samples revealed no other intestinal bacterial pathogens.

Six patients who yielded non-toxigenic isolates on culture gave positive results with lactoferrin tests. These positive tests were associated with infection (one each) with *Campylobacter*, *Salmonella* and *Shigella* spp., one ulcerative colitis, one gastrointestinal bleed and one bleeding duodenal ulcer.

## Discussion

*Clostridium difficile* is a Gram-positive, strictly anaerobic spore-forming rod. It is said to be carried in the intestine of up to 3% of the adult population. It may be found in the environment.<sup>12</sup> Carriage of the organism is more common in

the neonatal period, during which time up to 70% of neonates may be colonised but display no evidence of disease.<sup>1</sup>

The pathogenic role of the organism is related to the production of toxins A and B. Some strains also produce a third toxin (binary toxin) of unknown function.

The laboratory diagnosis of *C. difficile* infection depends on the detection of toxins in the faeces of a patient with diarrhoea while on antibiotics, or who has had exposure to antibiotics within the previous eight weeks. Traditionally, the use of tissue culture cell lines to detect the cytopathic effect of *C. difficile* cytotoxin (toxin B) followed by neutralisation of the effect with *C. difficile* antitoxin or *C. sordelli* antitoxin has been used as the definitive diagnostic test.<sup>11</sup> However, many diagnostic laboratories no longer have tissue culture availability. In addition, the test takes a minimum of 24 h to perform, is labour-intensive and has largely been superseded in the diagnostic laboratory by the more rapid and technically less-demanding ELISA tests (taking around 90 min) or flow-through (Ft) tests (taking approximately 25 min).

The current recommendation is that laboratories should use an ELISA or Ft test that detects toxins A and B, as some strains of *C. difficile* have a faulty toxin A gene and consequently only produce toxin B. These A–B+ strains are capable of causing disease and have been involved in outbreaks among hospitalised patients. Recent studies question the sensitivity of commercial faecal toxin assays<sup>4</sup> and the cell cytotoxin assay (especially in patients with severe CDI).<sup>13</sup> This study supports these findings, recovering 48 toxigenic isolates on culture, even though the initial single faecal toxin AB test was negative. This suggests that a proportion of patients who harbour a toxigenic strain of the organism would fail to be diagnosed using a single initial commercial test for faecal toxin. As this has treatment and infection control implications, the value of other non-culture methods to diagnose CDI was investigated.

**Table 3.** Utility of the GDH 'screening' test to detect all positive cultures and toxigenic cultures (1007 samples tested).

|              | All culture-positive | All culture-negative | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Toxigenic culture-positive | Toxigenic culture-negative | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|--------------|----------------------|----------------------|-----------------|-----------------|---------|---------|----------------------------|----------------------------|-----------------|-----------------|---------|---------|
| GDH-positive | 115                  | 8                    | 95.0            | 99.1            | 93.5    | 99.3    | 83                         | 40                         | 95.4            | 95.7            | 67.5    | 99.5    |
| GDH-negative | 6                    | 878                  |                 |                 |         |         | 4                          | 880                        |                 |                 |         |         |

Delmee *et al.* have shown that culture of faeces that were negative for toxin AB followed by toxigenicity testing of the isolate (toxigenic culture) increased their detection rate of infected patients with *C. difficile* by 3.4%.<sup>14</sup> Recently, surprise has been penned at the fact that diagnostic laboratories do not culture for toxin-producing *C. difficile* on those faecal samples that are toxin AB-negative from patients with acute diarrhoea in hospital.<sup>15</sup> The present study shows that, in the diagnostic laboratory, commercial kits for faecal toxin, when compared to toxigenic culture, have low sensitivity. This low figure is consistent with other reports<sup>16-19</sup> and is summarised in the recent review by Bartlett and Gerding.<sup>4</sup> It is generally accepted that cytotoxin assays using cell cultures are more sensitive but that they (as well as culture) are labour-intensive, costly and require technical expertise.

In the present study, an evaluation of *C. difficile*-specific GDH in a faecal sample shows that it is sensitive and specific for the presence and, more importantly, absence of the organism. Comparison of the performance of culture with GDH reveals that GDH testing has NPVs of 99.3% and 99.5% (all strains and toxigenic strains, respectively) and PPVs of 93.5% down to 67.5% (all strains and toxigenic strains, respectively). The test is an easy-to-perform kit Ft device and yields a result in approximately 25 minutes.

The GDH test can only be used to indicate presence of the organism in faeces; it does not provide information on toxigenicity. Faecal samples that yield a positive GDH test should be further tested for toxin AB production. However, a proportion of patients have a negative faecal toxin AB test result, even in the presence of a positive toxigenic isolate recovered by culture. Therefore, the role of a test for faecal lactoferrin to demonstrate the presence of intestinal inflammation in these patients was considered.

Previous studies show that patients with advanced CDI have significantly higher levels of lactoferrin in their faeces than those with mild disease,<sup>20</sup> and that the relationship between CDI and faecal lactoferrin is statistically significant.<sup>21</sup> The lactoferrin test was performed on those samples that were positive for GDH and/or toxin AB and which eventually grew a toxigenic isolate. This provided information on the presence of intestinal inflammation in patients who were known to harbour toxigenic *C. difficile*.

A positive faecal lactoferrin test cannot be interpreted in isolation, as other infectious and non-infectious causes of intestinal inflammation may also yield a positive result. Eighty patients who were faecal toxin AB-negative and lactoferrin-negative yielded *C. difficile* on culture (30 of which were toxigenic strains), which probably reflected colonisation. These observations have some interest as, at the very least, a large proportion of these patients may pose an infection control risk because they have diarrhoea and are shedding toxigenic strains into the environment.

Eighteen patients who were GDH- and culture-positive with a toxigenic strain were faecal toxin AB-negative and demonstrated a positive faecal lactoferrin test. This is an avenue for further investigation because some of these patients had clinically severe disease including antibiotic-associated colitis and pseudomembranous colitis.

Patients who present with clinical signs and symptoms of suspected CDI and yet have a negative toxin test should be monitored with other tests that may help clinical decision-making. Other workers support this view.<sup>22</sup> Severe disease is associated with considerable morbidity and mortality,<sup>23,24</sup> and

previously we have shown that some of these patients present with negative faecal toxin tests.<sup>25</sup>

Patients who have negative toxin tests yet have clinical evidence of CDI may fail to be treated, as discussed by Bartlett and Gerding.<sup>4</sup> Additional information gained by using the GDH and lactoferrin tests at the initial investigation stage (followed by toxigenic culture) could provisionally identify some patients with CDI who may need treatment but who have a negative initial faecal toxin test.

This work suggests that only those samples that are GDH-positive need to be tested subsequently for faecal toxin AB production. Samples that are GDH-positive but faecal toxin-negative should be sent for toxigenic culture. Recently, a two-step algorithm has been proposed by Fenner *et al.* using a test for GDH as a screen, followed by a faecal toxin test.<sup>26</sup>

This study concurs with the proposal by Fenner *et al.* and further suggests that a test for faecal lactoferrin could be included on selected samples to identify some patients with moderate to severe disease. Early indication of severe inflammation would be useful to guide appropriate clinical management.

## Conclusions

The current diagnosis of *C. difficile* infection relies on the demonstration of toxins A and/or B in the faeces of a diarrhoeal patient with a history of antibiotic exposure. However, it is now known that a single faecal toxin AB test may be diagnostically unreliable in a proportion of patients with CDI. The degrees of sensitivity of such kits also depends on the prevalence of *C. difficile* in an institution.

The use of a direct faecal GDH screen, together with a faecal toxin AB test, could improve the diagnosis of CDI. The utility of the GDH test depends on its ability to rule out the presence of the organism, as it closely parallels culture. The presence of both GDH and toxin confirms a diagnosis. Patients with a positive faecal GDH but negative toxin can be monitored closely while awaiting the result of a toxigenic culture.

Addition of a lactoferrin test may be useful to those clinicians who strongly suspect CDI in a patient who has a negative toxin test but a positive GDH test. The decision about whether or not to repeat faecal toxin AB tests can then be made on an individual basis. □

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