Detection of *Clostridium difficile* infection: a suggested laboratory diagnostic algorithm

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ABSTRACT

Currently, the diagnosis of *Clostridium difficile* infection (CDI) relies on the detection of toxins A and B in faeces but the sensitivity of these tests has been questioned, particularly in advanced disease. In this context, additional methods to enhance the diagnosis of *C. difficile* have been investigated. In this study, 1007 faecal samples are tested using toxigenic culture, an immunoassay for toxins AB and the *C. difficile*-specific glutamate dehydrogenase (GDH) test. Samples positive by any of the above tests are evaluated for the presence of faecal lactoferrin as an indicator of intestinal inflammation. Patients with evidence of inflammation but with negative toxin AB tests are followed up to assess clinical outcome. The toxin AB test was positive in 35 samples (3.4%), while 121 (12%) samples were culture-positive, 87 (8.6%) of which were toxigenic. Glutamate dehydrogenase proved to be a sensitive and specific marker of *C. difficile* with a negative predictive value of 99.3% (95% CI: 0.98–1.00). Faecal lactoferrin was positive in 52/129 (40.3%) samples tested. A cohort of 15 patients with a negative faecal toxin AB and a positive lactoferrin test was *C. difficile* culture-positive with a toxigenic isolate; clinically, all had advanced CDI. All demonstrated faecal toxin between five and 41 days later on repeat testing. It is suggested that a two-step algorithm be used to include screening faecal samples for GDH, with positive samples tested for faecal toxin AB and lactoferrin. Patients who present with a negative faecal toxin AB test and a positive lactoferrin test were serially tested for faecal toxin AB every five to seven days until a diagnosis was established. More sensitive tests than enzyme-linked immunosorbent assay (ELISA) for the detection of faecal toxin, or the use of a rapid specific test for the presence of a toxigenic strain, must be considered in such patients.

KEY WORDS: Antibiotic-associated diarrhea.
Clostridium difficile, toxins A and B.
Colitis.
Glutamate dehydrogenase.
Lactoferrin.
Toxigenic culture.

Introduction

Clostridium difficile is the most commonly implicated organism in antibiotic-associated colitis and is a major pathogen in healthcare settings.[1] The spectrum of disease varies from mild diarrhoea to pseudomembranous colitis and toxic megacolon. Clinical suspicion of *C. difficile* infection (CDI) arises when a patient who has had recent exposure to antibiotics develops diarrhoea. Currently, the mainstay of laboratory diagnosis, as recommended by the UK Department of Health, is the detection of *C. difficile* toxins A (enterotoxin) and B (cytotoxin) in the faeces by immunoassay methods,[2] mainly because the results are available the same day that samples are received in the laboratory. The detection of cytotoxin by cell cytotoxicity assay (CTA), followed by neutralisation of the cytopathic effect, however, is regarded as the gold standard and is more sensitive than commercial immunoassays.[3]

However, cytotoxicity assays have a minimum turnaround time of 24 h and are labour-intensive. Toxin tests by commercial immunoassays may be suboptimal if used alone due to the low sensitivity of commercial kits compared with CTA, or culture of the faeces followed by toxigenicity testing of the cultured isolate.[3–6] Many laboratories have abandoned the use of culture methods and rely heavily on commercial immunoassays for the detection of toxins. The major disadvantage of such a strategy is the failure to detect toxin in some patients early in their disease, resulting in a 'negative window' before diagnosis is established.[7] This is not just a phenomenon associated with commercial immunoassays as it has also been reported by workers using the CTA.[8]

The use of additional tests such as detection of glutamate dehydrogenase (GDH) and faecal lactoferrin have been reported previously as adjuncts to diagnosis of CDI. Glutamate dehydrogenase is a constitutive enzyme produced by all strains of *C. difficile*, independently of their toxigenicity. The detection of *C. difficile*-specific GDH has been used as an alternative to culture to determine the presence of the organism in the faeces with a high level of sensitivity.[4]

The presence of faecal lactoferrin has been reported to be a sensitive test for intestinal inflammation.[9] Other workers have shown that patients with advanced CDI have significantly higher levels of lactoferrin in their faeces than those with mild disease.[10,11] The authors of the present study recently published findings on the laboratory use of GDH together with toxigenic culture and faecal lactoferrin.[12]

The aim of this study is to evaluate the use of a combination of tests (GDH, faecal toxin AB immunoassay, culture and faecal lactoferrin) for the rapid and accurate diagnosis of CDI. This involves a two-step algorithm using GDH as the initial screen, followed by a test for faecal toxins AB and lactoferrin.

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Materials and methods

The study period was February to September 2008, during which a total of 1007 consecutive patients were evaluated, all of whom developed diarrhoea after being admitted to University College London Hospitals. All request forms indicated that infection with *C. difficile* was suspected. Available demographic data were recorded.

Liquid faecal samples submitted to the laboratory for *C. difficile* investigation were included in the study. Faecal samples that arrived in the laboratory more than 24 h after collection were excluded from the study.

All faecal samples (n=1007) were tested for the presence of toxins A and B using the Quik-Chek AB test kit (Techlab, Virginia, USA). In addition, all faecal samples were cultured according to published methods.[13] Culture of faeces, identification of isolates as *C. difficile* and toxigenicity testing of the isolates were performed as described previously.[12]

An aliquot of each faecal sample was also tested for the presence of *C. difficile*-specific GDH using the Quik-Chek kit (Techlab, Virginia, USA). Samples positive for GDH but negative on the routine faecal toxin AB test were further tested using five other commercial toxin test kits (Premier Immunocard and enzyme-linked immunosorbent assay [ELISA, Launch Diagnostics, UK]; Remel Xpect and Prospect [Oxoid, UK]; Techlab Quik-Chek AB II [Techlab]) to ensure that the result obtained from routine testing was consistent among other commercial ELISA assays.

Subsequently, all GDH-, toxin- or culture-positive faecal samples were tested for the presence of lactoferrin using the Eze-Vue kit (Techlab). The purpose of this test was to identify those patients with advanced CDI.

Strains of *C. difficile* cultured from 15 patients who were GDH- and lactoferrin-positive but faecal toxin-negative were ribotyped[14] to establish whether or not this finding was peculiar to a specific ribotype. The faecal toxin AB test was repeated on serial stool samples collected from these patients at intervals of five to seven days. Their clinical data and outcomes were also recorded.

All kit-based tests were performed according to the manufacturers' instructions with the appropriate controls.

Table 1. Demographic data for patients with suspected CDI.

Gender	Age group (years)	Toxigenic culture-positive No. (%)	Total (%)
Male	>65	25 (28.3)	246 (24.4)
	<65	25 (28.3)	279 (27.7)
Female	>65	22 (26.4)	244 (24.2)
	<65	15 (17.0)	238 (23.7)
		87	1007

Table 2. A comparison of *C. difficile* toxigenic culture with detection of faecal toxin AB by immunoassay for the diagnosis of *C. difficile* infection in hospitalised patients with acute diarrhoea.

		C. difficile toxigenic culture		
		Positive	Negative	Total
Faecal toxin AB test	Positive	35	0	35
	Negative	52	920*	972
Total		87	920	1007

*Includes 34 non-toxigenic isolates

Sensitivity: 40.23% (95% confidence interval [CI]: 0.30–0.51)

Specificity:100% (95% CI: 0.99–1.00)

PPV: 100% (95% CI: 0.88–1.00)

NPV: 94.65% (95% CI: 0.93-0.96)

Table 3. *C. difficile*-specific faecal GDH as a surrogate for the presence of the organism in faeces: a comparison with culture.

		C. difficile culture		
		Positive	Negative	Total
Faecal GDH	Positive	115	8	123
	Negative	6	878	884
Total		121	886	1007

GDH: Glutamate dehydrogenase (*C. difficile*-specific)

Sensitivity: 95.04 % (95% confidence interval [CI]: 0.89–0.98)

Specificity: 99.1% (95% CI: 0.98–1.00)

PPV: 93.5% (95% CI: 0.87–0.97)

NPV: 99.32% (95% CI: 0.98–1.00)

Results

Of the 1007 faecal samples tested, culture yielded 121 positive specimens, of which 87 were toxigenic isolates. This indicated a prevalence of the toxigenic organism of 8.6% in this patient cohort. Using the faecal toxin AB test, 35 samples were positive for *C. difficile* (prevalence: 3.4%). Demographic data of all patients with a diagnosis of suspected CDI, and their toxigenic culture results, are presented in Table 1.

Using the faecal toxin AB test alone, 35/87 patients harbouring a toxigenic strain of *C. difficile* would have been diagnosed with CDI (sensitivity: 40.2% [95% confidence interval {CI}: 0.30–0.51]) (Table 2).

The detection of GDH as a marker of *C. difficile* in the faeces was documented in 123/1007 faecal samples (12.2%). The sensitivity and specificity of GDH compared to culture for the detection of all strains of *C. difficile* is shown in Table 3.

A positive GDH test but negative routine faecal toxin AB test was found in 88 samples. Six of the 884 samples giving a negative GDH test grew *C. difficile* on culture, four of which were toxigenic.

The lactoferrin test was performed on all faecal samples positive for *C. difficile* toxins A and B, culture or GDH. Of the 129 samples tested, 52 (40.3%) were lactoferrin-positive (Table 4). Examination of the case notes of these 52 patients revealed that 46 were not currently diagnosed with other inflammatory conditions of the gastrointestinal tract (i.e., other intestinal infection, ulcerative colitis, Crohn's disease or inflammatory bowel disease [IBD]). Six patients with a positive lactoferrin test grew non-toxigenic isolates of *C. difficile*. Their positive lactoferrin was caused by *Campylobacter* infection, *Salmonella* infection, *Shigella*

infection, ulcerative colitis, gastrointestinal bleed and a bleeding duodenal ulcer.

In this study, 28 patients had evidence of advanced CDI as indicated by positivity for faecal toxin AB and lactoferrin. All were also culture-positive with a toxigenic isolate. Some patients (18 in total) with a negative faecal toxin AB and a positive lactoferrin test were also *C. difficile* culture-positive with a toxigenic isolate. Seven patients with a positive faecal toxin AB test were lactoferrin-negative. Of the 70 patients who were faecal toxin- and lactoferrin-negative, 62 were culture-positive (30 isolates were toxigenic). Eight patients were culture-negative.

Fifteen of the 18 patients who had a negative faecal toxin test, a positive faecal lactoferrin test and were *C. difficile* culture-positive with a toxigenic isolate were followed up to ascertain outcome and their isolates were ribotyped. These data are presented in Table 5.

Table 4. The use of faecal lactoferrin as an indicator of intestinal inflammation in patients culture-positive with toxigenic strains of *C. difficile*.

		Toxigenic *C. difficile*		
		Positive	Negative	Total
Faecal lactoferrin	Positive	46	6*	52
	Negative	41	36	77
Total		87	42	129

*All six patients had other positive reasons for lactoferrin (see text)

Discussion

The mainstay of the laboratory diagnosis of *C. difficile* infection in the UK is a positive toxin test on a single sample of diarrhoeal faeces, as recommended by current guidance.[2] Recent studies have questioned the sensitivity of the faecal toxin assay, especially in patients with severe CDI.[8] The present study supports this finding; 48 faecal samples grew toxigenic isolates on culture, although the initial single faecal toxin AB test was negative, suggesting that a proportion of patients harbouring a toxigenic strain of the organism would fail to be diagnosed (4.7% of all patients investigated). Of particular importance was that 18 of these 48 patients had a positive faecal lactoferrin, suggesting moderate to severe disease (39.1% of patients with severe CDI). As this has both treatment and infection control implications, the potential value of other non-culture methods to diagnose CDI was analysed.

Delmee *et al*. have shown that culture of faeces that were negative for toxin AB followed by toxigenicity testing of the isolate increased their detection rate of *C. difficile*-infected patients by 3.4%.[15] The findings of the present study concur with this figure. Fordtran has recorded his dismay at the fact that laboratories do not routinely culture for *C. difficile* (with a subsequent toxigenicity test on the isolate) on those faecal samples that are toxin AB-negative from patients with acute diarrhoea in hospital.[16] In the current authors' hands, when compared to toxigenic culture, the faecal ELISA toxin test showed a sensitivity of 40.2% (95% CI: 0.30–0.51). This low figure is consistent with other reports,[17–20] and is also summarised in a recent review by Bartlett and Gerding.[21] However, culture methods and cytotoxin assays are labour-

Table 5. Clinical and ribotype data on 15 patients* with initial negative faecal toxin AB tests.

Patient No.	Age (years)	Gender	Days to a positive test			Clinical details	Ribotype
			GDH	Lactoferrin	Faecal toxin AB (No. prior negative toxin tests)		
1	79	F	2	2	9 (1)	Colitis	027
2	63	M	2	2	11 (2)	Colectomy	027
3	77	M	2	2	35 (5)	Colectomy	001
4	71	M	2	2	41 (6)	Colitis	002
5	70	F	2	2	10 (2)	Severe diarrhoea	027
6	67	M	2	2	5 (1)	Severe diarrhoea	015
7	NA	M	2	2	33 (4)	Severe diarrhoea	027
8	87	F	7†	7†	23 (2)	Pseudomembranous colitis	002
9	57	M	2	2	24 (3)	Severe diarrhoea	NT
10	56	M	2	2	30 (5)	Severe diarrhoea	027
11	72	F	3	3	20 (3)	Colitis	027
12	63	F	2	2	15 (2)	Colitis	014
13	65	M	2	2	20 (4)	PMC	023
14	68	M	2	2	10 (2)	Colitis; colectomy	026
15	52	F	2	2	5 (1)	?Colitis	015

NT: not typable

*All patients were GDH-, lactoferrin- and culture-positive with a toxigenic strain

†The Day 2 sample was lost in transit; the Day 7 sample was the first one tested

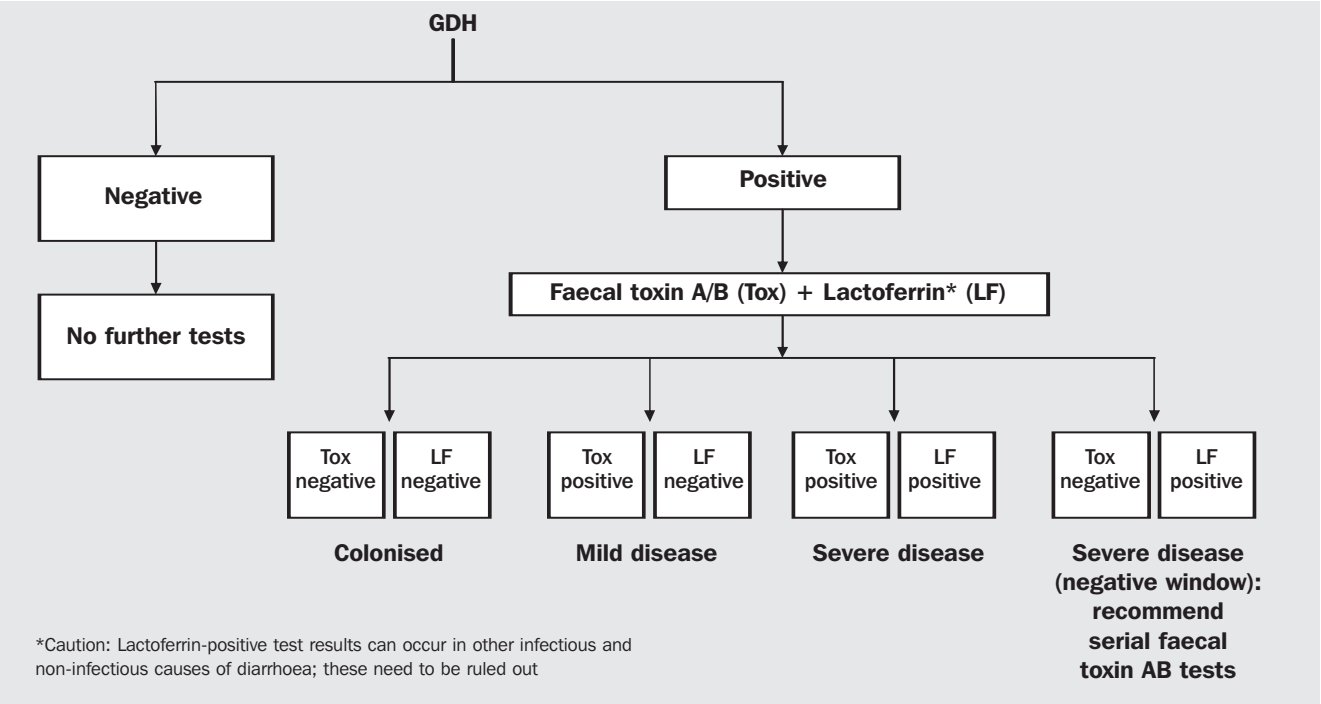

Fig. 1. Proposed two-step algorithm for the rapid and accurate diagnosis of clinically-suspected CDI with a hypothetical interpretation of the model.

intensive, costly and require technical expertise (i.e., reasons why many laboratories have stopped performing them).

This study evaluated a simple test that could be used as a screen to indicate the presence of *C. difficile* in the faecal sample. Furthermore, detection of *C. difficile*-specific GDH in a faecal sample is sensitive and specific for the presence of the organism. As shown in Table 3, comparison of the performance of GDH with culture found that it had a negative predictive value of 99.32% (95% CI: 0.98–1.00), a sensitivity of 95.4% (95% CI: 0.89–0.98) and a positive predictive value of 93.5% (95% CI: 0.87–0.97). The test is an easy-to-perform kit-based Pad ELISA and yields a result in approximately 20 min (a 96-well plate is also available for large workloads).

The GDH test can only be used to indicate presence of the organism in faeces, and does not provide information on toxigenicity. Faecal samples that yield a positive GDH test should be further tested for toxin AB production. However, this study has shown that a proportion of patients have a negative faecal toxin AB test result even in the presence of a positive culture with a toxigenic isolate. Therefore, the role of the faecal lactoferrin test to determine the presence of intestinal inflammation was evaluated in these patients.

It has already been shown that patients with advanced CDI have significantly higher levels of lactoferrin in their faeces than those with mild disease, and that the relationship between CDI and faecal lactoferrin is statistically significant.[10,11] The faecal lactoferrin test was performed on those faecal samples that were positive for GDH, culture or toxin, having already established the sensitivity of GDH compared with culture. This provided information on the presence of intestinal inflammation in patients known to harbour *C. difficile*.

A positive faecal lactoferrin test should not be interpreted in isolation, as other infectious and non-infectious causes of intestinal inflammation may also yield a positive result. Patients who were faecal toxin AB-positive but lactoferrin-negative could either have had mild disease or had diarrhoea due to another cause, with concomitant colonisation of the intestine with a toxigenic strain. In the patients who were faecal toxin AB-negative and lactoferrin-negative, it was possible to recover the organism on culture in 62 patients, with 34 of the strains being toxigenic. The authors believe that these 34 patients were either colonised with the organism or had mild disease not diagnosed by the use of a single faecal toxin AB test. These observations merit further investigation. However, these patients pose an infection control risk as they are shedding toxigenic strains into the environment.

There were 15 patients within the cohort who were GDH- and culture-positive with a toxigenic strain, were faecal toxin AB-negative and demonstrated a positive faecal lactoferrin test. Results of a detailed investigation of these patients are shown in Table 5. Serial faecal toxin AB tests were performed on these patients and all had demonstrable faecal toxin between five and 41 days after the initial negative faecal toxin test result. Explanations for this late toxin detection include the possibility that these patients had acquired a new infection with a different strain, or just the poor sensitivity of the ELISA tests to detect the presence of toxin. Clearly, this is an avenue for further investigation. All 15 patients had clinically confirmed advanced CDI either by the need for interventional surgery, sigmoidoscopy or computed tomography scans.

It is the authors' contention that patients who present with clinical signs and symptoms of suspected CDI may require serial faecal toxin tests every five to seven days until a diagnosis is established. Other workers support this view.[22] Fifteen isolates were ribotyped and six were identified as 027. Although the numbers are too small to draw any clinical or epidemiological conclusions, ribotype 027 is currently the most prevalent in the UK and has been associated with considerable morbidity and mortality.[23]

Patients who have negative toxin tests yet have clinical

evidence of CDI may fail to be treated, as discussed by Bartlett and Gerding.[21] Clearly, the additional information gained by using the GDH and lactoferrin tests at the initial investigation stage (followed by toxigenic culture) could identify with some confidence those who may need treatment. This not only instigates appropriate therapy but may also prevent spread of the disease.

A simple two-step algorithm is proposed and its hypothetical interpretation (Fig. 1) for the rapid and accurate diagnosis of CDI is based on a GDH screening test. Only those samples that are GDH-positive need to be tested subsequently for faecal toxin AB production. A two-step algorithm has been proposed recently by Fenner *et al*. using GDH as a negative screen followed by a faecal toxin test.[4] In addition, the authors suggest that a test for faecal lactoferrin could be included on all GDH-positive faecal samples to differentiate moderate to severe from mild disease. Early indication of severe inflammation would be useful to guide appropriate clinical management. Early work in the authors' laboratory comparing lactoferrin positivity with the severity score suggested by Zar *et al*.[24] indicates that those patients with a severity score of greater than two (indicating the likelihood of severe disease) have a positive faecal lactoferrin. Currently, this hypothesis is being tested in an ongoing study.

In conclusion, a single faecal toxin AB test may be unreliable in a proportion of patients with CDI, and further studies are needed in the UK to confirm that a testing protocol using faecal GDH as a screening method, followed by the faecal toxin AB and lactoferrin tests, would improve the diagnosis of CDI. It is also believed that those patients who present with a negative faecal toxin AB test and a positive lactoferrin test should be serially tested for faecal toxin AB every five to seven days to help establish a diagnosis.

Additional research on other useful tests such as real-time polymerase chain reaction (RT-PCR) for the *C. difficile* toxin genes should also be performed to overcome the low sensitivity of ELISA tests. The authors also support the contention of Delmee *et al*.[15] that all laboratories should be able to culture for *C. difficile* or be able to send faeces to a laboratory that has this facility. This is particularly important when toxin tests are negative on faecal samples from patients who have a possible clinical diagnosis of CDI. □

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