# Molecular test to determine toxigenic capabilities in GDH-positive, toxin-negative samples: evaluation of the Portrait toxigenic *C. difficile* assay

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**ABSTRACT**

New recommendations for testing and reporting of *Clostridium difficile* were introduced in the NHS in 2012. These guidelines have improved identification of potential *C. difficile* infection (CDI) cases, but questions remain around the management of glutamate dehydrogenase (GDH)-positive, toxin-negative patients. This study aims to assess the introduction of the Portrait *C. difficile* assay as the third step to identify the presence of the toxigenic *C. difficile* B (*tcdB*) gene and thus determine toxigenic capability. Stool samples with a GDH-positive, toxin-negative result were tested using the Portrait analyser to detect the presence of *tcdB*. A retrospective evaluation was performed, assessing the clinical course of patients who were isolated as a result of the current algorithm using GDH enzyme immunoassay (EIA) and toxin EIA. Of the stool samples tested, 40% carried the *tcdB* gene. Four *tcdB*-positive stool samples initially toxin A/B-negative subsequently became positive. Thirteen patients were isolated, four of which did not have the *tcdB* gene. The total time to 'process' a positive CDI case was 102 hours and cost £592. The additional time and cost of incorporating the Portrait toxigenic *C. difficile* assay was 105–115 minutes and £46.48 to £51.88. This study confirms that toxigenic capabilities in GDH-positive, toxin-negative specimens can facilitate effective treatment and infection prevention, and results show there is potential value in repeat toxin testing.

KEY WORDS: Clostridium difficile.
Glutamate dehydrogenase.
Nucleic acid amplification techniques.
Toxins.

## Introduction

Diarrhoea is a common complication of modern medical care. *Clostridium difficile* has been identified as a key mediator of antibiotic-associated diarrhoea and is implicated in 50–70% of antibiotic-associated colitis and over 90% of those with antibiotic-associated pseudomembranous colitis.[1]

In 2011 there were 2053 deaths involving *C. difficile* infection (CDI) in England and Wales, with 91.1% of deaths due to CDI occurring in NHS hospitals during 2009 to 2011.[2] Predisposing host factors and circumstances affecting the frequency and severity of disease include hospitalisation, advanced age, underlying illness, recent surgery, and recent administration of antibiotics or antineoplastic agents that possess antibacterial activity. Almost 15% of hospitalised patients receiving low-risk β-lactam antibiotics develop diarrhoea, and rates for those receiving high-risk cephalosporins and clindamycin range from 10% to 25%.[1]

The latest figures show that a total of 7670 NHS trust-apportioned cases of CDI occurred in patients aged two years and over in England between April 2011 and March 2012, a reduction from 10,417 in April 2010 to March 2011.[2] It has been calculated that the annual cost of CDI to an average-sized district general hospital is approximately £400,000, including 2100 lost bed days.[3] On average, a patient with a hospital-acquired infection spends 2.5 times longer in hospital than an uninfected patient.[4] Infected patients stay significantly longer than controls, averaging 21.3 days in hospital, including an average of 14 days in isolation.[5]

In March 2012, together with the Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection (ARHAI), the Department of Heath (DH) updated its guidance on the reporting and diagnosis of CDI. This update was provoked by a report produced in 2009 by Eastwood *et al.*,[6] which demonstrated the poor accuracy and effectiveness of available *C. difficile* testing kits.

The first 'gold standard' methods of *C. difficile* testing included cytotoxin testing and cytotoxigenic culture. However, results can take 24–72 hours. *C. difficile*-associated diarrhoea (CDAD) is a progressive disease, and severe colitis can occur within as little as five days; thus, there is a serious requirement to detect CDI at as early a stage as possible.

The study by Eastwood *et al.*[6] compared assays for the detection of *C. difficile* toxins A/B, glutamate dehydrogenase (GDH) and a real-time polymerase chain reaction (PCR) assay for the *C. difficile tcdB* gene, against previous 'gold standard' methods. All kits involved in the study showed low positive predictive value (PPV; 48.6–86.8%), thus compromising the clinical utility of single tests for the laboratory diagnosis of CDI. The optimum rapid single test was PCR for the toxin B (*tcdB*) gene, as this had the highest negative predictive value (NPV; 99.1%).

However, these studies demonstrated that higher PPV for toxigenic *C. difficile* can be achieved by first testing for GDH,

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a maintenance protein produced by toxigenic and non-toxigenic *C. difficile*, followed by a nucleic acid amplification test (NAAT) detecting toxin genes to confirm toxigenicity, rather than by testing for toxins alone. It was concluded that diagnostic algorithms which optimise test combinations for the laboratory diagnosis of CDI needed to be defined. As part of the updated guidance issued by the DH, a two-test protocol was introduced to deliver the most accurate results for CDI and provide the ability to categorise patients clinically.

Assays chosen to be incorporated into algorithms for identification of toxigenic *C. difficile* were based on the main options in use in the NHS at that time.[7] The two-test screening protocol consisted of a GDH enzyme immunoassay (EIA) or NAAT, followed by a 'sensitive' toxin EIA. If the first test (GDH or NAAT) is negative, the second test ('sensitive' toxin EIA) need not be performed. If the GDH EIA or NAAT is positive, and the toxin EIA is positive (PPV: 91.4%), then *C. difficile* is most likely to be present, and infective control measures are taken, and, if appropriate, alternative antibiotic treatment is arranged. The result is included in mandatory reporting to the Health Protection Agency (HPA). If the GDH EIA is negative (NPV: 98.9%) then *C. difficile* or CDI is considered very unlikely to be present, and the result is therefore not included in mandatory reporting. Other causes of diarrhoea are considered.

Uncertainty arises when patient specimens are shown to be GDH EIA-positive but toxin EIA-negative. Such strains of *C. difficile* present in these specimens may or may not be toxigenic. A major concern is whether or not these patients have the potential to spread toxigenic strains of *C. difficile* to others. Samples with evidence of the presence of *C. difficile*, but no demonstrable toxin, can indicate potential *C. difficile* excretors. This information may aid infection prevention and control measures.[7] It is then recommended that a third test (e.g., NAAT or PCR) be optionally added to the algorithm to identify samples as potential toxigenic *C. difficile* excretors.[7]

The present study aims to assess the introduction of the Portrait *C. difficile* assay as the third step to identify the presence of the toxigenic *C. difficile* B (*tcdB*) gene and thus determine toxigenic capability.

## Materials and methods

A total of 40 routine diagnostic stool samples sent to the microbiology laboratory at Colchester Hospital University NHS Foundation Trust (CHUFT) for CDI investigation (i.e., symptomatic patients) were tested. Specimens were assessed according to the Bristol stool chart and only those representing types 6 and 7 were examined. Thirteen aliquots of these samples were stored at –28°C for subsequent testing by the Portrait toxigenic *C. difficile* assay (Great Basin, Salt Lake City USA), with the remaining 27 samples tested directly from specimens as they were received. Frozen aliquots were thawed once and all samples were tested according to the routine two-step protocol for CDI, based on DH guidance. Two known GDH-positive and toxin-positive specimens were tested as positive controls and one known GDH-negative specimen was tested as a negative control.

### *Glutamate dehydrogenase EIA*

The primary test used to detect GHD was the Proflow *C. difficile* GDH test (Pro-Lab Diagnostics), which is used routinely in the CHUFT microbiology laboratory. The sensitivity and specificity of this test has been determined as 95.8% and 95.0%, respectively. The tests were performed according to the manufacturer's instructions by one laboratory assistant and validated by a biomedical scientist (Band 6).

### *Toxin EIA*

The second test in the *C. difficile* testing algorithm was the Quikchek Complete test (TechLab), which is used routinely in the laboratory for the rapid detection of toxin in faeces. The sensitivity and specificity of this test has been

**Table 1.** Clinical data for patients with positive GDH and negative toxin results.

| Clinical factors | | *tcdB*-positive (*n*=15) | *tcdB*-negative (*n*=22) |
|---|---|---|---|
| Fever | >38.5°C | 3 | 2 |
| Diarrhoea | >48 h | 13 | 14 |
| | <48 h | 1 | 1 |
| Melaena | | 2 | 0 |
| Abdominal pain | | 2 | 1 |
| Abdominal CT scans | | 2 | 0 |
| Colonoscopy | | 0 | 0 |
| Antibiotics | | 11 | 11 |
| Other current medication | | 5 | 7 |
| Chemotherapy/radiotherapy | | 1 | 5 |
| Surgery | | 5 | 6 |
| Gastric tube feeding | | 1 | 0 |
| Iron treatment | | 1 | 2 |
| Proton pump inhibitors and $H_2$ blockers | | 1 | 1 |
| Prior CDI | | 2 | 0 |
| Later positive toxin assay results | | 4 | 0 |
| Ward | Medical | 4 | 3 |
| | Obstetrics and gynaecology | 0 | 1 |
| | Orthopaedics | 0 | 1 |
| | Surgical | 2 | 0 |
| | ICU | 4 | 3 |
| | Care of the elderly | 3 | 4 |
| | GP* | 4 | 10 |
| | Isolation† | 1 | 0 |
| WBC | >15x10$^9$/L | 0 | 2 |
| | <15x10$^9$/L | 14 | 15 |
| | Unknown | 3 | 4 |
| CRP | <5 mg/L | 0 | 4 |
| | >15 mg/L | 13 | 13 |
| | Unknown | 3 | 5 |

*Including hospices.
†In isolation for reasons other than CDI (one case: pneumonia).
CDI: *C. difficile* infection, ICU: intensive care unit,
WBC: white blood count, CRP: C-reactive protein.

determined as 90.2% and 99.7%, respectively. The tests were performed according to the manufacturer's instructions by one laboratory assistant and validated by a biomedical scientist (Band 6).

***Nucleic acid amplification test***

The Portrait analyser toxigenic *C. difficile* test (Great Basin) uses a new technique for the detection of the *tcdB* gene in faeces. Extraction, amplification and detection of the target gene occur within the test cartridge once inserted into the Portrait analyser. The test is initiated and the analyser performs automated processing and resulting.

The principle of this assay relies on isothermal-blocked, primer-mediated, helicase-dependent amplification (bpHDA). In this process, biotin-labelled primers direct the amplification of target nucleic acid sequences within the toxigenic *C. difficile* pathogenicity locus (PaLoc) under isothermal conditions. These biotin-labelled, amplified target DNA sequences are hybridised to an array of probes immobilised on a silicon chip, and incubated with anti-biotin antibody conjugated to streptavidin-horseradish peroxidase (SA-HRP). Any unbound conjugate is washed away and tetramethylbenzidine (TMB) is added to produce a coloured precipitate at the location of the probe/target sequence complex. This results in the formation of coloured spots on the chip surface for automated visualisation by the Portrait analyser's optical reader. The software interprets the spot pattern and returns a result.

Time to result from test initiation is approximately 90 minutes. Sensitivity and specificity has been determined as 98.0% and 90.9%, respectively. These tests were performed according to the manufacturer's instructions, but the results were not included in any clinical reports.

***Clinical evaluation***

Demographic data, laboratory results and the clinical history of the patient were collected using the CUHFT laboratory information management system (LIMS). Current presentation and treatment were collected by consultant microbiologists in the department using a test evaluation case report form. Data collected included basis for admission, characteristics of current episode of diarrhoea, consistency (Bristol stool chart), melaena, duration and abdominal pain, C-reactive protein (CRP), white blood cell count (WBC), site of patient, and other factors significantly associated with colonisation and infection by hospital-acquired *C. difficile*; antibiotic therapy, other current medication, previous CDI, chemotherapy, immune status, surgery, abdominal computed tomography (CT) scans, iron treatment, gastric tube feeding, colonoscopy, previous hospitalisation, proton pump inhibitors and $H_2$ blockers, and later toxin assay results. The retrospective evaluation of the clinical course of patients isolated in wards or bays was performed to assess the significance of the NAAT result when considering implications of clinical outcomes and cost benefit.

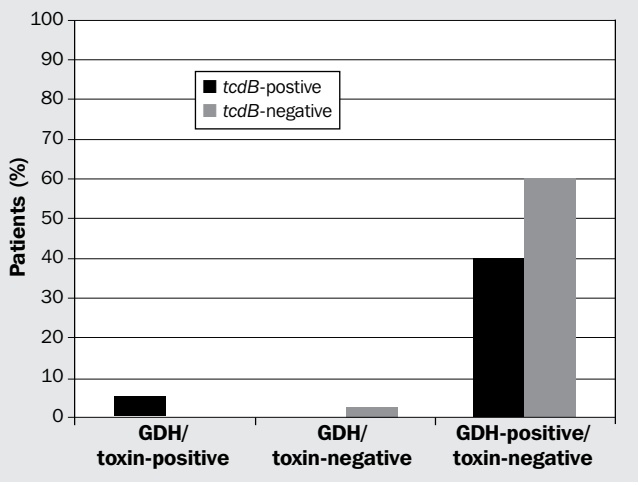


**Fig 1.** Test results for the different assays used on the 40 samples showing the percentages with and without the *tcdB* gene.

## Results

Results for the presence of the *tcdB* gene responsible for eliciting the enterotoxin B found in all toxigenic strains using the Portrait analyser are summarised in Figure 1. Of all GDH-positive, toxin A/B-negative stool samples ($n$=37), 40% were positive for *tcdB*. The clinical characteristics of patients in these two categories were compared. Previous episodes of CDI were detected in two patients with positive *tcdB* stools; none were detected in patients with negative *tcdB* stools. Similarly, positive toxin A/B test results found after the initial CDI screening were seen in four patients in the *tcdB*-positive category; none were detected in the *tcdB*-negative group. Clinical and laboratory data obtained for both groups are given in Table 1.

Follow up of clinical outcome and the infection control procedures taken in response to the reported CDI screening results was performed on all GDH-positive, toxin A/B-negative stools samples ($n$=37). Thirteen patients were moved to a side room or to the isolation ward. Four patients were *tcdB*-negative and these were eventually concluded not to have CDI. These patients were kept in isolated areas for two to nine days. One patient had no episode of diarrhoea but was isolated based on clinical observation, despite being toxin A/B-negative – a day after the patient was moved, vancomycin-resistant enterococci (VRE) was reported. Nine toxin A/B-negative patients were isolated, four of whom had a positive PCR result and were later positive for toxin A/B. A further four patients were deemed not likely to have CDI and all were moved back to their original wards. One patient had no particular risk factors associated with CDI. One patient was isolated because of a history of CDI and was known to be a *C. difficile* carrier.

## Discussion

Following the clinical course of patients isolated allowed assessment of the significance of the detection of the *tcdB* gene. Of the GDH-positive, toxin A/B-negative stool samples ($n$=37) tested, 40% were positive for *tcdB* (Fig. 1), which was consistent with the results found in trials of the Portrait toxigenic *C. difficile* assay in other selected UK laboratories (unpublished data).

One interesting observation was the change to positive toxigenicity in some patients. Positive toxin A/B test results found after initial CDI screening were seen in four patients in the *tcdB*-positive group but none in the *tcdB*-negative

**Table 2.** Serial laboratory data on three delayed toxin-positive patients.

| | WBC count (x10$^9$/L) | | |
|---|---|---|---|
| Days since CDI screen | Patient 5 | Patient 9 | Patient 11 |
| 1 | 5.6 | | |
| 2 | | | |
| 3 | 9.0 | | |
| 4 | 9.7 | | |
| 5 | | 10.1 | |
| 6 | 4.8 | 7.8 | 6.1 |
| 7 | | 18.8 | |
| 8 | | 12.1 | |
| 9 | | 6.0 | |
| 10 | 3.7 | 8.4 | |
| 11 | | 10.9 | |
| 12 | 7.5 | 11 | |
| 13 | | 12.8 | 13.5 |
| 14 | 7.2 | 12.9 | |
| 15 | | 18.3 | |
| 16 | | 17.1 | |
| 17 | | | |
| 18 | 8.2 | | |
| 19 | | | |
| 20 | | | 6.9 |
| 21 | | | |
| 29 | | | 5.8 |
| 33 | | | |
| 34 | 10.6 | | |
| 35 | | | |
| 36 | | | |
| 37 | 10.5 | | |
| 38 | 9.8 | | |
| 39 | | | |
| 40 | 16.0 | | |
| 41 | | | |
| 42 | | | |
| 43 | 17.1 | | |
| 44 | | | |
| 45 | 8.2 | | |
| 48 | | | |
| 49 | 9.1 | | |
| 50 | 8.1 | | |
| 56 | 8.5 | | |
| 60 | | | |
| 61 | 7.3 | | |

group. As noted earlier, a concern for NHS trusts is whether or not such patients have the potential to spread toxigenic strains of *C. difficile* to other patients. Presence of the *tcdB* gene in samples that are GDH-positive but toxin-negative can indicate potential toxigenic *C. difficile* excretors. Clinical monitoring of such patients should extend over several days.

One patient isolated with a GDH-positive, toxin A/B-negative and *tcdB*-positive result was treated with a proton pump inhibitor (PPI), which decreases gastric acidity. This patient may have been more likely than the others to contract *C. difficile*; however, PPIs as a risk factor for the development of CDI has not been demonstrated reproducibly in previous studies.[8,9] This patient was also treated with metronidazole, which is a recommended treatment for an initial episode or first recurrence of mild to moderate episodes of CDI.[10,11]

This evaluation demonstrates the advantages of NAAT in combination with the current GDH and toxin A/B protocol to identify potential excretors of toxigenic *C. difficile*. Three patients with toxin A/B-negative and *tcdB*-negative results were placed in isolation unnecessarily, incurring additional hospital costs; thus, negative NAAT results may lead to financial savings.

Four patients initially seen to be toxin-negative but NAAT-positive were isolated on the basis of the laboratory results (GDH-positive, toxin-negative) and clinical observation. These patients became toxin-positive during the course of their stay. In three patients there was a concomitant rise in WBCs, suggesting the development of CDI (Table 2).

In conclusion, this study demonstrates the benefit of using NAAT (Portrait toxigenic *C. difficile* assay) as a third test in the current algorithm to identify samples that come from potential *C. difficile* excretors and thereby aid infection prevention and control measures. A positive NAAT result may not be the trigger for treatment decisions but should be taken into consideration along with the clinical condition of the patient. Repeat toxin tests should be considered in the management of such patients. □

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