**Diagnosis and outcome of *Clostridium difficile* infection by toxin enzyme immunoassay and PCR in an island population**

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**Conflicts of interest**

SK has nothing to declare

RP has nothing to declare

IM has nothing to declare

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**Abstract**

**Backgrounds and Aims**

*Clostridium difficile* infection (CDI) is a potentially life-threatening cause of diarrhoea. Correct laboratory diagnosis is essential to differentiate CDI from other causes of diarrhoea. A positive faecal *C.difficile* toxin (CDT) is the best indicator of CDI, but the significance of a positive faecal nucleic acid amplification test (NAAT) remains unclear. Our aim was to elucidate the significance of CDI diagnostics in patients in Jersey.

**Methods**

A retrospective, 5-year study was conducted at an island district general hospital of patients who developed CDI. Patients were grouped according to CDT and NAAT status and their association with outcome (indicators of severity and 30-day case-fatality rate) compared.

**Results**

207 patients were toxin positive, 92 PCR positive and toxin negative and 39 had a stool sample negative by both toxin and PCR testing. A positive toxin stool sample was associated with both significantly higher white cell count (14.5 x 109/L vs. 11.3 x 109/L, p=0.003) and CRP (114.7 mg/dL vs. 82.9 mg/dL, p=0.001) but NAAT positivity was not (p=0.269, 0.728). A positive CDT assay was a significant independent predictor of death (OR:1.89 [95%CI:1.04-3.43], p=0.046) but a positive NAAT in CDT negative samples was not (OR: 1.02 [95% CI: 0.34-3.12], p=1.0).

**Conclusions**

The findings of this study, derived from evolving clinical practice, provide greater clarity in the interpretation of current CDI diagnostics. In CDT negative disease, a positive NAAT neither predicts disease severity nor mortality. PCR positive, toxin negative patients require instigation of infection control measures but the need for specific treatment remains unclear.

**Introduction**

*Clostridium difficile* is a gram-positive, anaerobic, spore-forming bacillus which causes antibiotic-associated diarrhoea and colitis, and in the most severe cases, pseudomembranous colitis, by the production of toxins A and/or B.[1](#_ENREF_1) *Clostridium difficile* infection (CDI) is recognised as a leading cause of infectious diarrhoea, particularly in hospitalised patients receiving antimicrobial therapy.[2-4](#_ENREF_2)(Bartlett 2002) CDI is reported as a cause of death in around 3,000 cases annually in the United Kingdom and up to 20,000 in the USA.[3-5](#_ENREF_3) There is an attributable case fatality rate of 6%-17%.[6-9](#_ENREF_6)

Consistently reported risk factors leading to CDI include antibiotic use as well as advanced age and severe underlying illness.[2-4](#_ENREF_2) As there are no specific clinical features that distinguish CDI from other causes of diarrhoea,[10](#_ENREF_10) rapid and accurate diagnostic tools are essential, not only to ensure prompt initiation of appropriate antibiotics to treat CDI, but also for timely institution of infection control measures to prevent transmission.[11](#_ENREF_11)

The cell-cytotoxicity assay, which detects neutralisable CDT, and, cytotoxigenic culture, which cultures *C. difficile* isolates then confirms toxin production *in vitro*, are considered gold standards for diagnosis.[4](#_ENREF_4), [12-15](#_ENREF_12) However, both tests are relatively expensive and time consuming to perform and are rarely used in routine laboratories. Commercially produced enzyme immunoassays (EIAs) for CDT, glutamate dehydrogenase (GDH) and nucleic acid amplification test (NAAT) are commonly used in diagnostic laboratories for rapid diagnosis. However, the performance of toxin EIAs is sub-optimal with positive predictive values as low as 50% in populations where disease prevalence is low[16](#_ENREF_16), [17](#_ENREF_17) and they are not recommended as standalone tests. Assays detecting GDH, a common enzyme and antigen produced by *C. difficile* and encoded by the *gluD* gene,*,* can be used as an initial screening tool to accurately rule out the presence of *C. difficile* in stool samples[18-20](#_ENREF_18) but poor specificity also makes it unsuitable as a standalone assay.[21](#_ENREF_21) Concerns that patients with CDI investigated by conventional toxin testing were being missed prompted many laboratories to adopt molecular testing as part of their diagnostic algorithm.[19](#_ENREF_19), [22](#_ENREF_22) NAAT is rapid and highly sensitive and concerns about the lower sensitivity of EIA testing [19](#_ENREF_19), [22](#_ENREF_22) has led to increased usage in hospitals worldwide, despite being more expensive.[23](#_ENREF_23) However, this methodology detects the DNA of the toxin gene of *C. difficile* rather than the presence of toxin in stool samples. As the tests for *C.difficile* detect different targets it is not surprising the results of different assays for *C. difficile* do not agree in all samples.[4](#_ENREF_4), [15](#_ENREF_15) The majority of large studies on *C. difficile* diagnostics show CDT EIA is the best predictor of outcome with NAAT tests providing no additional information about disease severity. [4](#_ENREF_4), [24](#_ENREF_24), [25](#_ENREF_25) Nonetheless, NAAT has improved clinical predictions in some studies [26-31](#_ENREF_26), so there is a need to examine the utility of NAAT testing across a number of different diagnostic settings.

A 2-stage diagnostic protocol for *C. difficile* was adopted in Jersey General Hospital in 2010, with the addition of GDH assay to CDT EIA testing.[32](#_ENREF_32) A further third stage incorporating NAAT was added to the diagnostic protocol later in 2010 after a cluster of *C. difficile* infection (Figure 1) and a desire to improve the sensitivity of the testing protocol. This process was audited after the introduction of these new testing methodologies.

The aim of this study was to compare the clinical and biochemical characteristics, and outcome of patients diagnosed with CDI by toxin EIA with those found to have a negative toxin test but positive by NAAT. We also assessed whether there were specific factors associated with a positive NAAT stool sample.

**Materials and methods**

The study protocol was approved by the hospital ethics board as part of an ongoing local audit of CDI patients.

*Design and setting*

This 5-year retrospective study was performed in the Medical Microbiology laboratory of the Jersey General Hospital, which serves a population of about 100,000 people. Jersey, the largest of the Channel Islands, is an island measuring 9 miles by 5 miles, which lies about 12 miles off the coast of France, in the English Channel. As the only microbiology laboratory on the island, all stool samples are tested in one location, providing an invaluable opportunity to monitor CDI throughout the island, in both the hospital and the community.

*Microbiological testing*

Between 2008 and 2012, liquid stool samples were tested for CDT and glutamate dehydrogenase (GDH) by membrane enzyme immunoassay (MEA) using the C. DIFF QUIK CHEK COMPLETE (Techlab, Blacksburg, V.A, USA). Before the change in diagnostic protocol in 2010, samples were tested for CDT only (Techlab, Blacksburg, V.A, USA). Testing was carried out in accordance with the manufacturer’s instructions. In patients found to be CDT positive, further testing was not repeated for 30 days. After the introduction of 3-stage testing, stool samples that were GDH positive but CD toxin negative, had a NAAT performed for the *C. difficile* toxin gene tcdB (GeneXpert *C. difficile*, Cepheid, USA). From receipt of the stool sample in the laboratory, 2-stage testing was typically performed within 1-2 hours. The third stage of the diagnostic protocol, NAAT, was processed within a further 2 hours.

*Data collection*

Clinical data were collected as follows: patient demographics, blood test results within 3 days of diarrhoeal stool samples (white cell count (WCC), C-reactive protein (CRP), serum creatinine, estimated glomerular filtration rate (eGFR) and serum albumin), dates of admission and discharge, antibiotic prescription in the previous 28 days and 30-day all-cause mortality. All data records were anonymised.

*Statistical analysis*

For statistical analysis, we used Stata 12 (Stata Corp, College Station, Texas, USA). Data range and logic checks were performed. Patients were grouped according to the permutation of their test results and univariate analysis performed, with the toxin EIA and NAAT test results used as predictors. Fisher’s exact test and unpaired *t*-tests were used as appropriate and statistical significance defined as p < 0.05.

**Results**

Between 2008 and early 2010, before the routine use of GDH EIA in Jersey, 974 stool samples were tested for toxin EIA alone of which 91 were positive (9.34%). GDH EIA testing was introduced in February 2010 to complement toxin EIA assessment. Of the 3,532 samples tested, 247 were GDH positive (6.99%). Of these, 116 were also found to be toxin EIA positive (3.28%). No GDH negative samples were toxin EIA positive. Following the introduction of 3-stage testing in late 2010, 131 stool samples found to be GDH positive but CD toxin negative, were subjected to further testing by PCR, with 92 found to be positive (70.23%). A summary of the results and testing methodologies is provided in table 1.

The patient cohort was divided according to CD toxin and NAAT status (Table 2). Age, sex, and co-morbidities were comparable between CDT positive and negative patients. Patients with a positive toxin EIA were more likely to have received antimicrobial therapy in the preceding 28 days (p < 0.0001).

Patient demographics were similar in the NAAT positive and negative groups. Hospital admission related to a gastrointestinal infection was more common in those with a positive molecular test (p = 0.004). Surgery of the gastrointestinal tract was more prevalent in the NAAT negative cohort (p = 0.003), although this is unlikely to be of clinical relevance. The rate of antibiotic prescription was similar in both NAAT positive (90.2%) and negative (71.8%) patients (p = 0.123).

*Biochemical parameters*

A positive toxin EIA stool sample was associated with both significantly higher WCC (14.5 x 109/L vs. 11.3 x 109/L, p = 0.003) and CRP (114.7 mg/dL vs. 82.9 mg/dL, p = 0.001). Renal function was worse in patients with a toxin positive test (eGFR 68.1 vs. 75.7 ml/min/1.73m2,p = 0.041). There was no significant difference in serum albumin between toxin EIA positive and negative patients. NAAT status was not associated with a difference in WCC, CRP, serum albumin or renal function.

*30-day case fatality*

21.3% of the toxin positive group died within 30 days compared to 12.5% in those with a stool sample negative for toxin. 30 day-mortality was 13% and 12.8% in NAAT positive and negative patients, respectively. A positive toxin EIA test was a significant independent predictor of death (OR: 1.89 [95% CI: 1.04 – 3.43], p = 0.046) but a positive NAAT in the absence of EIA-detectable toxin was not (OR: 1.02 [95% CI: 0.34 – 3.12], p = 1.0)

**Discussion**

In this study we compared the clinical and biochemical characteristics of patients with a positive toxin EIA test to those with a toxin negative but positive NAAT stool sample. The clinical features of patients with a positive toxin EIA result were significantly different from those with a negative toxin stool sample. A positive toxin stool sample was associated with raised white cell count and CRP, deterioration in renal function and increased 30-day case fatality. In toxin negative patients, a positive NAAT test result did not correlate with increased inflammatory markers and was not a univariate predictor of 30-day case fatality. We found no association between antibiotic exposure in the previous 28 days and a positive NAAT test, with no single antimicrobial agent implicated.

There has been debate over the clinical relevance of a positive NAAT stool test in the context of toxin negative stool sample. Smaller earlier studies showed that NAAT detected more positive cases than CDT EIA and was assumed to be more sensitive.[26-31](#_ENREF_26) These studies found no difference in clinical features of CDT and NAAT positive patients. A subsequent large, multi-centre, prospective study considered 12,420 liquid stool samples.[4](#_ENREF_4) Toxin positivity, detected by the reference method of cytotoxin assay, correlated with clinical outcome. In contrast, cytotoxigenic culture and NAAT positivity did not predict mortality or disease severity, assessed by white cell count, rise in plasma creatinine and changes in serum albumin. Similarly, Polage *et al* [*24*](#_ENREF_24) noted that patients who were *C difficile* negative by toxin EIA but positive by NAAT testing had significantly less severe diarrhoea at the time of testing, more rapid resolution of diarrhoea, fewer CDI-related complications and less mortality (*p* < 0.001) when compared with patients who were positive by stool toxin and NAAT. The clinical presentation and outcomes of patients who presented with toxin negative, PCR positive disease were no different from those that were negative by both toxin EIA and PCR.

Our study corroborates these findings in a “real-life” setting as part of evolving clinical practice, adding to the accumulating evidence that unlike toxin positivity, a NAAT test is not a univariate predictor of disease severity or mortality.[4](#_ENREF_4), [24](#_ENREF_24), [25](#_ENREF_25), [33-35](#_ENREF_33)

Notwithstanding that NAAT appears to be a poor prognostic marker in CDI, patients with toxigenic strains of *C. difficile* indicated by a positive molecular test should be considered as potential spore excretors.[4](#_ENREF_4) In these circumstances, infection control measures, including isolation, should be considered until the stool is of normal consistency.[36](#_ENREF_36) Molecular tests benefit from a rapid turnaround time, highlighted by studies reporting that NAAT reduced the time taken for CDI specific therapy to be prescribed as well as minimising the use of empirical therapy in patients without CDI.[37](#_ENREF_37)

We found application of a 3-step algorithm, incorporating GDH and CD toxin testing followed by NAAT in those stool samples that were GDH positive but CD toxin negative, to be a practicable method of identifying individuals who were NAAT positive. We found it prudent and feasible to isolate these patients, ensuring adherence to strict infection control measures. The introduction of NAAT testing, strict compliance to the local antibiotic policy and the introduction of hydrogen peroxide vapourisation in December 2010 was associated with termination of the CDI outbreak, and a year-on-year reduction in toxin positive disease. A recent Canadian study investigated the effect of detecting and isolating *C. difficile* asymptomatic carriers at hospital admission on the incidence of health care-associated CDI (HA-CDI).[38](#_ENREF_38) Admission screening was conducted by detecting the *tcdB* gene by PCR on a rectal swab, with positive patients placed under strict contact isolation precautions during their inpatient stay. They reported a statistically significant reduction in the HA-CDI incidence rate following this intervention with no change in the incidence of community-acquired and ambulatory care-associated CDI.

Previous work has not evaluated whether recent antimicrobial therapy may contribute to the development of a NAAT positive stool sample. We found no association but definitive conclusions cannot be made from our modest sample size (NAAT positive, n = 92, NAAT negative, n = 39). NAAT positive patients may be carriers of toxigenic *C.difficile* with the potential to cause cross-infection, but their diarrhoea is probably due to an alternative aetiology.[4](#_ENREF_4)

Our study is limited by its retrospective design, with the potential for the introduction of bias. It was performed in a single centre and coincided with a small outbreak of CDI. Our practice was restricted to just one commercially available kit to perform PCR so caution must be exercised when extrapolating these data to other populations and assays. As PCR tests were repeated infrequently, we are unable to confirm continued carriage of *C. difficile* in these patients, although a strong possibility remains. In practice, repeat PCR testing is rarely useful unless there is evidence of a new infection.[39](#_ENREF_39)

In conclusion, NAAT appears to have no role in predicting disease severity or mortality. In contrast, the presence of *C. difficile* toxin in the stool, as assessed by EIA, is a strong univariate predictor of worse biological markers of disease and poor outcome. Infection control measures should be instituted for NAAT positive toxin negative patients but the role of antibiotic prophylaxis, particularly in those at high risk, remains to be elucidated. Further prospective studies are needed to more clearly define the clinical need to treat these patients.

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**Figure 1.** *Clostridium difficile* infection diagnosed in Jersey by year

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | GDH | CDT | NAAT | Total  |
| 1-stage | ND | + | ND | 91/974 |
| 2-stage | - | ND | ND | 3285/3532 |
| 2-stage  | + | - | ND | 131/3532 |
| 2-stage  | + | + | ND | 116/3532 |
| 3-stage  | + | - | - | 39/131 |
| 3-stage | + | - | + | 92/131 |

**Table 1.** Combinations of results obtained in the study population.

1-stage testing with CDT testing only (2008-2009)

2-stage testing with GDH and CDT (Early 2010)

3-stage testing with GDH, CDT and NAAT if indicated (From late 2010)

GDH, Glutamate dehydrogenase; CDT, *C. difficile* toxin; NAAT, Nucleic acid amplification test

ND = not done

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  (CDT +)n = 207 | (CDT -)n = 144 |  (NAAT +)n = 92 | (NAAT -)n = 39 | **CDT + vs. CDT -** **p-values** | **NAAT + vs. NAAT -p-values** |
| Age in years (SD) | 76.6 (14.6) | 73.5 (16.4) | 72.3 (17.0) | 74.1 (16.5) | 0.064 | 0.581 |
| Sex, M/F | 88/119 | 62/82 | 41/51 | 14/25 | 1.000 | 0.440 |
| **Comorbidities** |  |  |  |  |  |  |
| Cardiovascular disease (%) | 23 (11.1) | 24 (16.7) | 15 (16.3) | 6 (15.4) | 0.152 | 1.000 |
| Chronic lung disease (%) | 83 (40.1) | 56 (38.9) | 38 (41.3) | 12 (30.8) | 0.826 | 0.362 |
| Cerebrovascular disease/dementia/immobility (%) | 40 (19.3) | 28 (19.4) | 17 (18.5) | 7 (17.9) | 1.000 | 1.000 |
| Diabetes (%) | 27 (13.0) | 30 (20.8) | 18 (19.6) | 8 (20.5) | 0.057 | 1.000 |
| Chronic GI disease (%) | 57 (27.5) | 50 (34.7) | 30 (32.6) | 17 (43.6) | 0.159 | 0.240 |
| Malignancy (%) | 32 (15.5) | 25 (17.4) | 14 (15.2) | 8 (20.5) | 0.661 | 0.454 |
| Alcoholic/liver disease (%) | 16 (7.7) | 13 (9.0) | 8 (8.7) | 4 (10.3) | 0.696 | 0.750 |
| Skin disease (%) | 17 (8.2) | 12 (8.3) | 10 (10.9) | 2 (5.1) | 1.000 | 0.508 |
| Musculoskeletal disease (%) | 21 (10.1) | 14 (9.7) | 11 (12.0) | 2 (5.1) | 1.000 | 0.343 |
| GI surgery (%) | 8 (3.9) | 12(8.3) | 6 (6.5) | 4 (10.3) | 0.100 | **0.003** |
| Orthopaedic surgery (%) | 14 (6.8) | 12 (8.3) | 7 (7.6) | 3 (7.7) | 0.680 | 1.000 |
| GI infection (%) | 23 (11.1) | 14 (9.7) | 12 (13.0) | 2 (5.1) | 0.727 | **0.004** |
| Lung infection (%) | 75 (36.2) | 46 (31.9) | 33 (35.9) | 8 (20.5) | 0.426 | 0.101 |
| UTI (%) | 56 (27.1) | 38 (26.4) | 29 (31.5) | 7 17.9) | 0.903 | 0.136 |
| Skin infection (%) | 17 (8.2) | 12 (8.3) | 10 (10.9) | 2 (5.1) | 1.000 | 0.508 |
| Previous MRSA (%) | 18 (8.7) | 15 (10.4) | 11 (12.0) | 3 (7.7) | 0.583 | 0.553 |
| **Antibiotics** |  |  |  |  |  |  |
| Antibiotic exposure in previous 28 days | 185 (89.4) | 23 (16.0) | 80 (86.7) | 29 (71.8) | **< 0.0001** | 0.123 |
| **Biochemical parameters** |  |  |  |  |  |  |
| Mean white cell count x 109/L (SD) | 14.5 (11.5) | 11.3 (5.1) | 11.6 (5.3) | 10.5 (4.5) | **0.003** | 0.269 |
| CRP mg/L (SD) | 114.7 (86.0) | 82.9 (82.3) | 86.6 (81.0) | 80.9 (88.2) | **0.0009** | 0.728 |
| Albumin g/dL (SD) | 29.4 (6.9) | 29.57 (7.5) | 29.2 (7.5) | 29.7 (6.8) | 0.825 | 0.788 |
| eGFR ml/min/1.73m2 (SD) | 68.1 (30.5) | 75.7 (31.5) | 77.5 (30.4) | 74.1 (33.5) | **0.041** | 0.581 |
| **Clinical characteristics** |  |  |  |  |  |  |
| Length of hospital stay days (SD) | 42.1 (86.2) | 33.1 (36.7) | 37.6 (40.2) | 24.4 (24.5) | 0.267 | 0.067 |
| 30-day case fatality (%) | 44 (21.3) | 18 (12.5) | 12 (13.0) | 5 (12.8) | **0.046** | 1.000 |

**Table 2.** Patient demographics, clinical characteristics and 30-day case fatality stratified by *C. difficile* toxin and NAAT.

CDT, *C. difficile* toxin; NAAT, nucleic acid amplification test.