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Enzyme immunoassay for *Clostridium Difficile* Infection: Once, Twice or Thrice? Is the Falling Incidence of *Clostridium Difficile* an Artefact?

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Abstract

Many clinical laboratories rely on stool enzyme immunoassays (EIAs) to diagnose *Clostridium difficile* infection (CDI). The test is limited by its modest sensitivity which has led to repeat testing. This study examines the impact of repeat stool testing in suspected CDI and describes clinicians' patterns of ordering tests.

In this retrospective study, analysis was performed on a database of toxin EIAs submitted to our laboratory for suspected CDI. For each patient, results of (up to) the first three stool samples, submitted in the first episode of diarrhoea, was analysed. Patterns of ordering tests were examined.

4,987 patients submitted 8,408 stool samples during the study period. Overall, 13.8% of EIAs were positive for *C.difficile*. Of these, 68% were diagnosed on the first test, 22% on the second and 10% on the third. 9.4% of first test samples were positive, rising to 14.8% for samples tested twice and 17.7% for samples tested thrice. Repeat testing increased CDI prevalence by 50%. Those testing negative for CDI on the first test were more likely to get re-tested compared to those testing positive (25% vs. 16.8%, [$\chi 2$= 15.8, $p<0.0001$]).

The prevalence of CDI increased under the three test policy using EIA justifying the need for repeat sample testing. Clinicians were more likely to request repeat tests if the initial result was negative. The implementation of single test rules in some trust will result in a fall in the CDI rate by a third. More work is needed to assess the impact of new two-stage testing policies.

Keywords: Clostridium difficile; Enzyme immunoassay; Repeat stool testing; Antibiotic associated diarrhoea

Introduction

Clostridium difficile infection (CDI) is the primary cause of antibiotic-associated diarrhoea and pseudomembranous colitis, with a reported mortality of 6-15% [1]. Risk factors for CDI include antibiotic usage, age, and hospitalisation. Among antibiotics, common causes include quinolones, clindamycin and cephalosporins [2-6]. Infection may lead to a spectrum of disease ranging from being asymptomatic to life-threatening pseudomembranous colitis [2]. Timely and accurate diagnosis is imperative to commence effective treatment, but also in order to institute appropriate infection control measures [7].

The gold standard (GS) tests [8] in the diagnosis of CDI include cell culture cytotoxin assay (CCNA) with reported sensitivities of 94-100% and a specificity of 99% [8] and toxigenic culture (TC) with reported sensitivities of 93% and a specificity of 93% [4,9] . However, these tests are time consuming and labour intensive to perform. Thus, many clinical laboratories rely solely on toxin enzyme immunoassay (EIA), as it provides a speedy result and is cheaper than CCNA and TC. A recent survey in England demonstrated that up to 70% of trusts were using EIA as a stand-alone test for CDI [10]. However, the advantage of speed is offset by the low sensitivity (63 to 99%) of EIA [11].

A report by the Health Protection Agency (HPA) in 2009 [4] found that EIAs may miss approximately 1 in 5 to 1 in 10 cases of CDI. Thus, many clinicians order repeat stool sample testing following a negative result, in an attempt to increase the diagnostic yield. Frequently, up to three EIAs are ordered to 'rule out' CDI [12]. Previous guidelines did not specify the optimal number of tests in the diagnostic workup of CDI [11,13]. Furthermore, EIAs have a sub-optimal positive predictive value (PPV) [4] leading to false positives, although the consequences of this, are less well appreciated.

However, recent published data has questioned the merit of a policy of repeat stool testing for CDI [7,11-16]. The latest guidance from the Department of Health state that EIAs alone are insufficient for the diagnosis of CDI [4]. A two-stage testing approach which includes a glutamate dehydrogenase (GDH) EIA as an initial screening test followed by a toxin EIA or CCNA for GDH positive samples has been recommended.

Polymerase Chain Reaction (PCR) assays are another test used to detect *C.difficile* toxin B gene from loose stool samples. *C. difficile* PCR has been reported to detect up to 35% more *C. Difficile* positive specimens than are detected with EIAs [17]. Kvach et al. reported their PCR assay to have a sensitivity of ≥ 91% versus EIA with a specificity of 95% and negative predictive value of 99% [17].

The primary objective of this study was to investigate the impact of repeated stool sample testing using toxin EIA, following an initial

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negative result, in suspected CDI. The secondary objective was to examine clinicians' patterns of ordering tests for suspected CDI.

Methods

We defined a retrospective cohort by analysing a database of all stool EIAs submitted to our microbiology laboratory for suspected CDI between January 2007 and September 2008. Samples analysed were from two large teaching hospitals (Bristol Royal Infirmary and Royal United Hospital, Bath) and from primary care. At that time, the laboratory used the Premier EIA (Meridian Bioscience Inc, Cincinnati, OH) to diagnose *C.difficile* in patients with diarrhoea. The test detects *C.difficile* toxins A and B. This kit has a published sensitivity of 91.7% (95% CI 84.7-96.1) and a specificity of 97.1 (95% CI 95.1-98.4), and a sensitivity of 80.8% (72.3-87.3) and a specificity of 97.5% (97.9-99.8) relative to CCNA [18].

For each patient, the results of the first three stool samples submitted in the first episode of diarrhoea were analysed. An episode of diarrhoea was defined as a 10-day period from the time the first sample was received; samples collected more than 10 days from the first one were not considered to be part of the same episode. Only the first episode of diarrhoea per patient was analysed. Thus, up to the first three samples of diarrhoea per patient were analysed; subsequent samples within an episode, or from later episodes, were disregarded. This was done to minimise confounding which may occur with subsequent episodes following an initial episode of *C. difficile* diarrhoea. It was not feasible to obtain the clinical details for each patient due to the large sample size and the retrospective nature of this study. Patients with a previous positive *C. difficile* toxin EIA test were excluded from the cohort. Our study was based on the assumption that a positive result indicated a clinical diagnosis of CDI; whilst potentially flawed, as the toxin EIA is not the gold standard, it reflects the information upon which clinicians have to act.

For each sample, results were coded as positive, negative or not ordered. Results were grouped into 14 different scenarios and were analysed using an approach similar to that adopted by Nemat [11].

Finally, the study examined physicians' ordering patterns of EIAs for suspected CDI across the study period.

Results

Laboratory data from 4987 patients were analysed; they had given 8408 stool samples within 10 days of the first sample (1.68 samples per patient). The number of patients with positive EIA results was 470 of 4987 (9.4%) for the first assay (Figure 1). The number with at least one positive test based on both the first and second test was 14.8%, while if this prevalence were based on a policy of testing three samples, it would

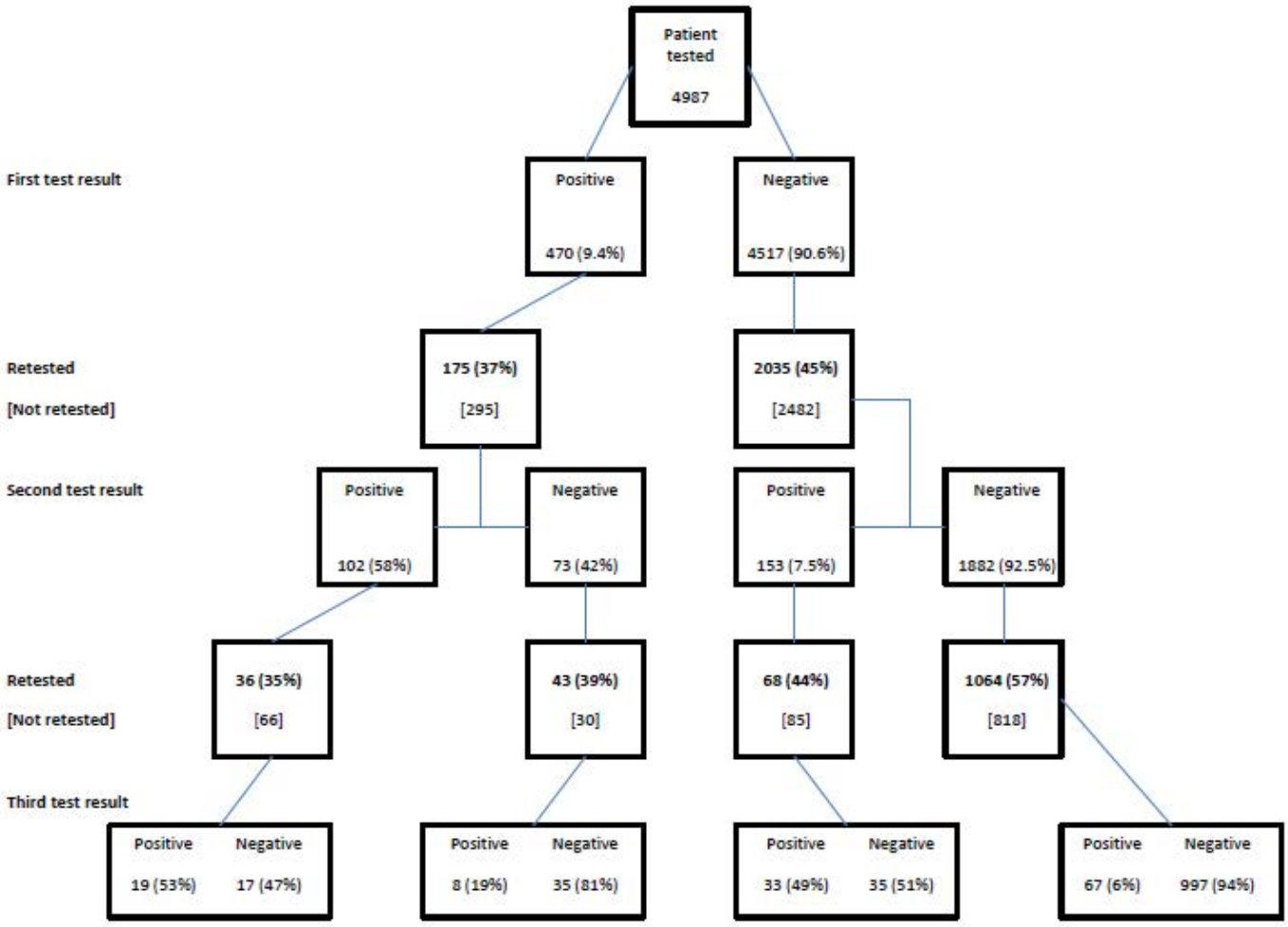

Figure 1: Result and re-testing of patients with possible CDI.

be 17.7%.Overall, 690/4987 (13.8%) patients tested positive within the first three tests, of these 68% were diagnosed on the first test, 22% on the second and 10% on the third (Figure 2 and Table 2).

In our cohort (Table 1), a single stool sample was ordered for suspected CDI in 2777 patients (55.6%); of these 295 (10.6%) had a positive result. However, 2210 patients had at least one further test; repeat testing was significantly more likely if the first test was negative (2035, 45% vs. 175, 37% for a positive result, $\chi 2= 10.5$, p=0.001) (Figure 1).

Of patients undergoing a second test (2210), 255 were positive. Of the 175 that had been positive, only 102 (58%) were re-test positive. A third test was performed for 79 patients that had initially tested positive, 36 (46%) were positive on second test and 43 (54%) were negative; amongst these a further repeat test was more likely in those whose second test was negative (39% vs. 36%, $\chi 2=9.6$, p=0.002).

Of these patients who were negative on the first test, 1882 of 2035 who were retested remained negative (92.5%), while 153 (7.5%) became positive on the second test. Further repeat testing was more likely in the patients who had had two negative tests (57%) compared with those were negative but became positive (44%) ($\chi 2=8.4$, p=0.004).

Overall, 25.1% of patients with a negative first test went on to have two more tests, while just 16.8% of those who were first test positive had two further tests ($\chi 2= 15.8$, p<0.0001, OR for repeat test if first test was negative was 1.4, [95% CI 1.14 to 1.68]; OR for third test if first was negative was 1.67, [95% CI 1.29 to 2.13]).

Discussion

Toxin EIA is widely employed in the investigation of CDI because of its low cost and rapid turnaround. In this study we have found that there is merit in repeat testing for CDI using EIA, in patients suspected to have this infection. As few as 68% of all positive cases were picked up on the first sample; a second sample found 22% more patients and the third added another 10%. This observation assumes that a positive EIA test means that the patient has CDI. We did not study the impact of tests performed after the third test as the number achieving more than three tests in 10 days was quite small.

There is a disincentive to repeat testing: Trusts have to reduce the number of repeated cases of CDI in line with targets set by the Department of Health. Failure to meet targets results in Trusts being fined. Logically, the number of positive results will increase if the EIA is repeated. We have shown that the prevalence of CDI increases from 9.4% to 13.8% if three samples are tested. This raises the possibility that the fall in CDI cases nationally is a result of a single test policy. However, our data also show that even though the repeat testing rate rose in the second half of the study the overall prevalence fell, marginally.

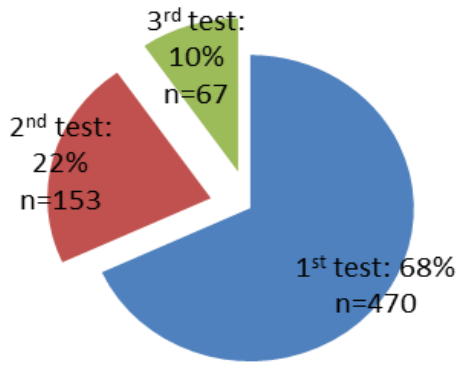

Figure 2: Number of EIAs needed to diagnose each positive *C.difficile* case.

Test 1	Test 2	Test 3	Frequency	Percentage
1. Negative	Not ordered		2482	49.8
2. Negative	Negative	Negative	997	20
3. Negative	Negative	Positive	67	1.3
4. Negative	Negative	Not ordered	818	16.4
5. Negative	Positive	Negative	35	0.7
6. Negative	Positive	Positive	33	0.7
7. Negative	Positive	Not ordered	85	1.7
8. Positive	Not ordered		295	5.9
9. Positive	Negative	Negative	35	0.7
10. Positive	Negative	Positive	8	0.2
11. Positive	Negative	Not ordered	30	0.6
12. Positive	Positive	Negative	17	0.3
13. Positive	Positive	Positive	19	0.4
14. Positive	Positive	Not ordered	66	1.3

Table 1: All tests ordered for presumptive CDI (n= 4987).

Test 1	Test 2	Test 3	Frequency	Percentage
1. Positive	Positive	Negative	67	9.7
2. Positive	Negative	Not ordered	85	12.3
3. Positive	Negative	Positive	35	5.1
4. Positive	Negative	Negative	33	4.8
5. Negative	Not ordered	Not ordered	295	42.8
6. Negative	Positive	Not ordered	30	4.4
7. Negative	Positive	Positive	35	5.1
8. Negative	Positive	Negative	8	1.2
9. Negative	Negative	Not ordered	66	9.6
10. Negative	Negative	Positive	17	2.5
11. Negative	Negative	Negative	19	2.8

Table 2: Tests ordered for patients with at least one positive result (n=690).

In this study, repeat testing was performed at clinicians' discretion. The local hospital policy was to perform a second test if the first test was negative, but the clinical suspicion remained high. Often, the decision to re-test was made by the nursing staff, with multiple samples being received. This may have influenced the results by adding more positive results to those patients. The 'SHEA position paper' appeared to suggest that repeat testing would increase the yield of CDI cases, but noted that it would not be cost-effective [19]. The suggestion arose from the report of Aronsson et al. [20] in which the second test added 7% more cases and a third 10%.

However, recent papers [7,11-16] have suggested that repeat testing should be avoided. Drees et al. [12] investigated the three sample rule using EIA testing. They reported that 85% of patients were diagnosed on the first test and attributed the improvement of the Aronnson et al. results [20] to newer generation EIAs. Cardona [14] found only 2.6% initially negative patients to become positive on repeat testing within 10 days of the first test. Nemat et al. [11] analysed a large database and found that 89.8% of cases in their series were found to be positive on the first test, 8.2% more were added on the second test and just 2% on the third. A retrospective analysis by Deshpande et al. [16] demonstrated a similar outcome. However, each study employed a different EIA, with variable specificities and sensitivities, which makes comparisons between the studies more difficult.

We also investigated the apparent impact of a positive, or negative, test on repeat testing. At the time the study data was collected; repeat

sampling was permitted in our institution. The apparent message from our data is that clinicians are more likely to repeat the investigation after a negative result is obtained than after a positive result: this suggests that clinicians do not 'trust' the negative result. However, this may also reflect a policy of notifying the ward by telephone immediately when a *C. difficile* test is positive, while simply issuing a negative result in the normal way: the latter thus has less impact on the staff requesting the stool sample.

A further observation is that some tests are reported to be negative after being positive once (43 patients, scenarios 9 and 10; Table 1) or even twice (17 patients, scenario 12; Table 1). This must cast some doubt on the EIA results - the negative result might be assumed to be a false negative, particularly when the negative result then 'becomes' positive again (8 patients, scenario 10; Table 1). Scenarios 9-14 (175 patients, Table 1) demonstrate situations where clinicians have ordered repeat tests after an initial positive result. The reasons for this could be that the result of the first sample being not yet available to the requesting clinician. Some clinicians wrongly assume that subsequent stool samples need to be sent following a positive result, to check for CDI clearance following treatment and this may account for the additional stool samples.

This study was done at a time where the dominant clone of *Clostridium difficile* in the Trust was the 027 strain [21]. As this produces more toxins then a false negative is again a little difficult to accept. Since then, the Clostridium Difficile Ribotyping Network (CDRN) [21] has observed a marked decrease in the prevalence of *C.difficile* 027 ribotype with rates dropping from 55% in 2007/8 to 12.4% in 2010/11. This may in part be due to the success of infection control measures introduced in the hospital [21]. A compensatory increase in other strains has been observed.

A recent paper [22] compared *C.difficile* detection methods using the same toxin EIA machine as that used in this study. They found the EIA had a specificity of 97.8% and a false positive rate of 2.2%. Even if testing only true negative cases, a repeat test will still generate false positives each time a re-test is done. In the case of this EIA, the predicted number of false positives is ~20 per 1000. Likewise with a sensitivity of 78.6%, if only true positives were tested, there would still be a large number of false negatives. It is possible therefore that the anomaly in results we have found in this study could be due to the reported sensitivity and specificity of the EIA.

However, at the time of the study, there were no alternative methods for diagnosing CDI in the laboratory, yet it is not unreasonable to suggest that a sample that gave inconsistent results is more likely to be incorrect on the single occasion when the result differed from the other two tests.

Following the update of guidelines on CDI diagnosis [4], our laboratory testing policies have been revised. The new algorithm includes an initial screening GDH EIA followed by toxin EIA on the GDH positive samples. Repeat samples on negative GDH EIA patients will be rejected in light of its high negative predictive value (NPV) (96.1%) [23]. However, new problems are likely to emerge in situations where samples test positive for GDH EIA but negative for toxin EIA due to its PPV (83.1%) [23]. Our primary institution (Bristol Royal Infirmary) has adopted a three stage algorithm. This includes a primary GDH EIA screen with negative samples receiving no further testing. GDH EIA positive samples then get a toxin EIA. If both tests are positive, this is interpreted as being positive for *C.difficile*. Discordant samples (GDH EIA positive but toxin EIA negative) are then tested by PCR. Those who are PCR positive are described as colonised and clinicians are advised to isolate patients until symptoms resolve. This testing algorithm provides a clearer understanding to the clinicians as to whether their patients are positive for *C.difficile* or not, particularly in those discordant samples, where the index of suspicion for CDI remains high. The downside to this algorithm includes additional costs and time.

Our study has several limitations. This study is an observational cohort in which the number of tests a patient received would have been based on many clinical factors, such as severity of symptoms, risk factors for CDI and probability of an alternative diagnosis such as Norovirus. Due to our sample size, we were unable to investigate the clinical scenario for each patient. Our analysis was based on the assumption that a positive EIA meant that the patient has CDI. We do not know what proportion of those cases testing positive were false positive results.

A policy of testing three samples appears to increase the yield of positive results, increasing the prevalence by 50%. The proportion of 'false positives' for these tests remains unclear. Further investigations using the gold standard tests- CCNA or TC to confirm the presence of toxigenic *C.difficile* should be carried out, to provide a more accurate diagnosis.

Conclusion

The prevalence of CDI increased under the three test policy using toxin EIA, justifying the need for a policy of repeat sample testing, although this is based on the assumption that diagnosis by EIA confirms CDI. Clinicians and nursing staff were more likely to ask for a repeat test after a negative EIA result than after a positive result, which may suggest a distrust of the EIA diagnosis compared to the observed clinical symptoms. The impact of new two-stage testing on the prevalence of CDI, and the need for repeat testing must be investigated in future.

Statement of Contributorship

Irasha Hettiarachchi conceived the study, participated in data analysis and drafted the manuscript

Martin Williams participated in data collection and reviewed the manuscript

Rosemary Greenwood was involved in data analysis Chris Probert participated in study design, data analysis and drafting of the manuscript

Natalie Evans and Alastair Strachan were involved in reviewing and drafting the manuscript

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