

Detection of clostridium difficile toxin in the stool biology essay

[Science](#), [Biology](#)



**ASSIGN
BUSTER**

By Hannah Akor This project is submitted in partial fulfillment of the requirements of the BSc (Hons) Degree in Biomedical Science University of Greenwich Medway Campus Chatham Maritime Kent ME4 4TB. UK

Declaration

" I confirm that the material presented in this project work is the result of my own efforts and that where material is derived from other authors / investigators has been used, it has been acknowledged in the text of the document and listed in the reference section"

Signed:

Name:

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Supervisor:

Abstract

Should be no more than one page. The abstract should say: Why you did what you did, how you did it, what you found out and what you concluded. You start page numbering from here - 1, 2 and so on.

This project is about the testing protocol for the detection of the bacterium "Clostridium difficile", in particular its toxin-producing spore. The results were obtained first by Enzyme immunoassay for C. difficile glutamate dehydrogenase antigen using various stool samples of patients at Medway Hospital. The samples positive for the antigen were then tested for the C. difficile toxin using Enzyme immune assay for C. difficile toxins A and B. The information of the patients from whom the samples were taken was very limited. Only their ages were known but not their gender or nationality or any of their medical history.

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Chapter 1: Introduction

1. 1 Outline of Project

The aims of laboratory diagnostic procedures in Clostridium difficile investigation are to detect the presence of the toxin producing bacterium in stool sample of a patient in hospital having frequent loose stools, abdominal pain, fever, and/or nausea during or following a course of antibiotics or following a recent gastrointestinal surgery. A full method of

1. 2 Clostridium difficile

1. 2. 1 History and Bacteriology

Clostridium difficile, also known as C. difficile or C. diff, is an obligate (Fraser and Swiencicki, 2013) strictly anaerobic, spore-forming, gram-positive rod (Madan and Petri, 2012). There are strains of C. difficile can either be toxigenic or non-toxigenic but only the toxigenic form causes disease and this is the strain that produces large protein toxins A (TcdA) and B (TcdB) (Jeffery et al. 2010). Toxins A and B are similar in structure (45% homology)

and are released during the late phases of the vegetative organism, it is thought probably at the time of cell lysis (Champoux et al. 2004). They disrupt proteins involved signal transduction, in particular, those that involve the actin cytoskeleton. This bacillus was first described and isolated in 1935 as a component of the faecal flora of healthy new-borns (Agzew, 2013) by Hall & O'Toole who gave it the name *Bacillus difficilis* which reflects the difficulties they had met in the process. The role of this organism and its toxins in pseudomembranous colitis and antibiotic-associated diarrhoea was discovered in the late 1970s. Its extensive presence was shown in environmental sites such as mud, soil, and sand, and in a wide variety of animal dung from cows to camels and donkeys. It was not until 1978 that *C. difficile* was implicated as the causative agent of antibiotic-associated diarrhoea and PMC. 1974 is the year the " *C. difficile* era" began, when high rates of pseudomembrane changes among patients at Barnes Hospital, in St. Louis, MO, were reported (Bartlet, 2008). These patients were receiving the drug clindamycin. This was the first study in which patients with antibiotic-associated diarrhoea undertook endoscopy as a routine diagnostic procedure for (Lyerly, 1995). The study was carried out by Tedesco et al. 1974. The original description of *C. difficile* cytotoxin was provided by Green, 1974, when they found a cytopathic toxin in the stools of guinea pigs treated with penicillin, which was previously thought to be due to a latent virus but in retrospect was probably *C. difficile* cytotoxin. *C. difficile* and its toxins were eventually identified as the causative agent of clindamycin-associated colitis due to a rapid succession of papers published between May 1977 and May 1978. Currently, it is the predominant cause of antibiotic-associated colitis

(Reller et al. 2007), specifically Pseudomembranous Colitis. *C. difficile* toxins A and B are regulated by *tcdR*, an alternative RNA polymerase sigma factor. This was described in 2001 and in 2007, the negative toxin regulator function of *tcdC* was elucidated through identification of the 18bp deletion and frame shift in *tcdC* in the hyper-virulent epidemic strains of *C. difficile*. Still to be elucidated are the roles of sporulation regulation and surface protein expression and adherence as likely pathogenesis factors in *C. difficile*. During the past 30 years, *C. difficile* infection has become one of the most common nosocomial, or hospital- acquired, infections in the US and a major cause of antibiotic-associated diarrhoea and pseudomembranous colitis in hospitalized patients and patients in long-term care facilities (Madan and Petri, 2012)

1. 2. 2 Epidemiology

The widespread and indiscriminate use of broad-spectrum antibiotics places patients at risk for *C. difficile* diarrhoea or colitis and has actually changed the epidemiology of CDI. This has been characterized by the emergence of a hyper virulent strain of *C. difficile* (BI/NAP1/027) and an increasing risk of treatment failure and recurrent infection (Lowy et al. 2010). For epidemiological studies, the bacteria can be typed by molecular DNA analyses, including protein electrophoresis, PCR and immunological tests (Groschel and Toye, 1996). Barlett and Gerding, 2008 state that *C. difficile* spores in the environment is highly prevalent among hospitals and facilities that offer long-term care so there is no surprise patients in these facilities

have higher rates of *C. difficile* colonization compared with healthy adults in the general population.

1. 3 Disease

The spectrum of disease ranges from a self-limiting mild diarrhoea to the advanced and severe illness characteristic of pseudomembranous colitis. Clinical disease with *C. difficile* toxin is the cause of 20–25% of all antibiotic associated diarrhoea and has historically been associated with prior antibiotic exposure and (Libby, 2009). The antibiotics most frequently implicated in predisposition to *C. difficile* infection are fluoroquinolones, clindamycin, cephalosporins, and penicillins (LaMont, 2013). Up to 50% of infants may be colonised in the first few months of life. Disease caused by *C. difficile*, however, is rarely present at this age.

1. 3. 1 Clostridium difficile-associated Diarrhoea (CDAD)

C. difficile is the most common identifiable cause of AAD in the hospital setting (Tung et al. 2009). Almost all drugs with an antibacterial spectrum of activity have been implicated causally in AAD. The most frequently implicated drugs are those which have a marked effect on the micro flora of the colon. These include broad spectrum beta lactams, cephalosporin's, clindamycin and fluoroquinolones (Impallomeni et al. 1995). The incidence of *C. difficile* infection has been shown to decrease once antibiotic therapy is controlled. In addition to *C. difficile* (Hogenauer et al. 1998), infection with *C. perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, *Candida* species and *Salmonella* species have been implicated with AAD (Asha et al. 2006).

1. 3. 2 Pseudomembranous colitis (PMC)

Other forms of Clostridium difficile-associated disease (also CDAD) have become recognized, including recurrent Clostridium difficile-associated diarrhoea, toxic megacolon, C difficile-associated arthritis, and septicaemia (McFarland et al. 1999).

1. 3. 3 Risk factors for disease

Nosocomial CDI is mainly a disease of older individuals (mainly those aged 65 and above) (Ahsa et al. 2006). They are the people that are most at risk of C difficile diarrhoea, possibly because their protective bacteroides diversity is more likely to be affected by antibiotics, which then permit growth of C difficile (Tonna and Welsby, 2005). Also, those undergoing general surgery¹⁷, oncology patients^{18, 19} and those with chronic renal disease²⁰ are at particular risk of infection by C. difficile Hopkins, M. and Macfarlane, G. (2002). Changes in predominant bacterial populations in. Journal of Medical Microbiology. 51 (5), 448-454. Hopkins, M., Sharp, R. and Macfarlane, G. (2001). Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut. 48 (2), 198-205.

1. 3. 4 Transmission of disease

1. 4 Pathogenesis

During periods of health, bacterial flora suppresses growth of C. difficile in the colon (Aas and Bakken, 2003). Broad spectrum antimicrobials have the potential to disrupt the balanced ecology of the stool flora, creating an opportunity for C. difficile overgrowth and attendant production of toxins C.

difficile colonizes the human intestinal tract (Bignardi, 1998) after the normal gut flora has been disrupted by indiscriminate use of antibiotics and chemotherapeutic agents and causes antibiotic-associated colitis. A spectrum of manifestations is observed (LaMont, 2013)The pathogenicity locus of *C. difficile* contains the *tcdC* gene which encodes the putative negative regulator of toxin A and B production (Spigaglia, and Mastrantonio, (2002). Base pair deletions (especially 18 bp) in the *tcdC* gene of the " epidemic" strain were initially thought to be responsible for the toxin hyperproduction (McDonald, et al. 2005; Spigaglia, and Mastrantonio, 2002)It has recently been reported that a single-nucleotide mutation at position 117, causing a frameshift that introduces a stop codon resulting in the truncation of the *tcdC* gene product, is the more likely mechanism (Sloan et al. 2008).

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1. 4. 1 Virulence factors

At least three potential virulence factors have been described; enterotoxin (toxin A), a cytotoxin (toxin B) and an apparently distinct motility-altering factor (Cascinu, 1995). The production of the two toxins A (enterotoxin) and B (cytotoxin) causes the distinctive mucosal damage consisting of plaque-like lesions leading to the formation of a pseudomembrane. *C. difficile* - toxin A is an enterotoxin and when it is produced, it stimulates the production of chemokines and thus attracts leukocytes. Toxin B is a cytotoxin, which causes distinctive cytopathic effects in cultured cells. Both toxins are glucosyl transferases and are part of a pathogenicity island that is not found

on the chromosomes of non-pathogenic strains of *C. difficile* (Kumar et al. 2010).

1. 4. 2 Immune response

Table 1. List of innate and adaptive immune responses to *C. difficile*

1. 5 Diagnosis

1. 5. 1 Clinical diagnosis

The classic clinical manifestation of *C. difficile* antibiotic associated diarrhoea is fever, diarrhoea, and leucocytosis occurring several days to months after antibiotic exposure (Libby and Bearman, (2009). Clinical features expressed in patients with antibiotic-associated PMC include watery diarrhoea (90-95%), bloody diarrhoea (5-10%), fever and leucocytosis. After 5-10 days of antibiotic therapy, symptoms generally begin to occur, however, they may occur as early as the first or second day or as late as 3-4 weeks after therapy is terminated (Cascinu, 1995). An endoscopic procedure can be used to diagnose *C. difficile* colitis. A gastroenterologist (a medical professional who specialises in disease of the digestive tract) can observe and biopsy any characteristic pseudomembranous lesions that may be present.

1. 5. 2 Laboratory diagnosis

The diagnosis should be based on results from a laboratory diagnostic test detecting toxin-producing strains (via enzyme immunoassay or polymerase chain reaction) or an endoscopic evaluation that demonstrates pseudomembranes in the colon. The use of polymerase chain reaction (PCR) for diagnosis of *C. difficile* infection (CDI) is favoured, either alone or as part

of an algorithm (including initial enzyme immunoassay [EIA] screening for glutamate dehydrogenase [GDH], with or without EIA screening for toxins A and B) (LaMont, 2013). A variety of risk factors affect the patient's vulnerability to CDI. These do include advanced age, hospitalisation and antibiotic treatment (Kumar et al. 2010). Relapse or reinfection develops in 10 to 25% of treated C. difficile cases, and patients may experience several episodes of relapsing colitis. Relapse may present within days or weeks of completing treatment for C. difficile; the clinical presentation may be similar to or more severe than the initial presentation. Diagnosis of CDI is usually confirmed by testing a stool sample for the presence of C. difficile toxin. Currently there is debate over the optimal testing strategy due to concerns having previously been raised regarding the reliability of many of the rapid CDT detection methods (Swindells et al. 2010). There are a number of tests available. These include: Polymerase chain reaction (PCR) Enzyme immunoassay (EIA) for C. difficile glutamate dehydrogenase (GDH) Enzyme immunoassay (EIA) for C. difficile toxins A and B Cell culture cytotoxicity assay Selective anaerobic culture LaMont, 2013 outlines these testing methods as follows:

- Polymerase chain reaction (PCR)

These tests, in real-time, detect toxin A and B genes and are highly sensitive and specific. The sensitivity of PCR comparable to that of the cytotoxicity assay and is greater than enzyme immunoassay. The results of PCR testing can be available within as little as one hour. Some do favour use of PCR in an

algorithm together with other assays such as EIA for GDH and EIA for toxins A and B, given its high sensitivity and potential for false positive results.

- Enzyme immunoassay (EIA) for C. difficile glutamate dehydrogenase (GDH)

All C. difficile isolates constitutively produce GDH antigen which is an essential enzyme. Detection of GDH antigen cannot distinguish between toxigenic and non-toxigenic therefore, testing for GDH antigen is useful as an initial screening step in a multistep approach, which also consists of subsequent testing with more specific assays such as PCR on specimens that are GDH antigen positive. GDH antigen testing is highly sensitive, and results are available in less than one hour.

- Enzyme immunoassay (EIA) for C. difficile toxins A and B

Most C. difficile strains produce both toxins A and B, although some strains produce either toxin A or B only. The sensitivity of EIA for toxins A and B is about 75% and the specificity is high (up to 99 %). For the test to be positive, 100 to 1000pg of toxin must be present making the false negative rate relatively high. A number of inexpensive assays are commercially available, and test results are available within hours. If the initial EIA test is negative, repeat testing is generally discouraged because the value of repeating the test is limited. Three studies found fewer than 10% more cases by repeated testing, while two others found 19% and 20% more cases with one or two further tests.

- Cell culture cytotoxicity assay

The cell culture cytotoxicity assay is the 'gold standard' test for diagnosis of *C. difficile* according to LaMont, 2013, and is the standard against which other tests should be compared. It is performed by adding a prepared stool sample (diluted, buffered, and filtered) to a monolayer of cultured cells. If *C. difficile* toxin is present, it exerts a cytopathic effect characterized by rounding of fibroblasts in tissue culture. The cytotoxicity assay is more sensitive than enzyme immunoassays, but is labour intensive and takes approximately two days.

- Selective anaerobic culture — Culture on selective medium with toxin testing of isolated *C. difficile* is the most sensitive diagnostic method, although it cannot distinguish toxin-producing strains from non-toxin producing strains. Prior treatment with heat or alcohol to select spores is sometimes used to improve yield. Culture is useful for epidemiologic studies, but is generally too slow and labour-intensive for clinical use. The suggested testing protocol according to Health Protection Scotland is to carry out an initial test. This will be a toxin immunoassay or PCR for toxin B gene or GDH test. At this stage negative results can be issued as final reports. It is not clinically useful to test asymptomatic patients. Therefore, it is particularly important to limit testing to those patients who exhibit the clinical symptoms of CDI including watery or loose diarrhoea three times a day or more and those who have risk factors of CDI such as recent or current antibiotic therapy, age, and concomitant illnesses. It is also important to test only diarrhoea specimens (those specimens that take the shape of the container). It may be requested when an outpatient develops these symptoms within 6-8 weeks after taking antibiotics, several

days after chemotherapy, or when a patient has a chronic gastrointestinal disorder that the doctor suspects is being exacerbated by a *C. difficile* infection. The CDT test may be requested to help diagnose the cause of frequent diarrhoea when a patient has leucocytes and/or blood in the stool and no other discernible cause has been detected i. e. parasites or other pathogenic bacteria. Another reason for this test to be requested is to monitor the effectiveness of treatment for *C. difficile* diarrhoea and to detect a recurrence although it is possible to detect the presence of *C. difficile* by actually growing the bacteria (stool culture). The results of the culture take several days and further testing must be done to detect the toxin. A positive *C. difficile* toxin test is indicative of the patient's diarrhoea being caused by an overgrowth of toxin-producing *C. difficile*. Occasionally, false positives may be seen with grossly (visibly) bloody stool samples. If the test is negative but the diarrhoea continues, another sample needs to be tested. The *C. difficile* toxin test does not detect 100% of cases and the toxin may have been missed the first time. Since the toxin breaks down at room temperature, a negative result may also indicate that the sample was not processed promptly or stored correctly prior to processing. A negative test result may also mean that the diarrhoea and other symptoms are being caused by something other than *C. difficile*. The customary method to detect *C. difficile* toxin A and B is by a rapid enzyme immunoassay. Depending on the test, the results are available after 1-4 hours. A more sensitive method to detect toxin is a cytotoxin test that looks for the toxic effects of stool on human cells grown in culture, but it requires 24-48 hours to get the result.

1. 6 Treatment

Since *C. difficile* was recognized as the cause of CDI in the late 1970s, metronidazole and vancomycin have been the primary treatment options (Gerding et al. 2008). According to Aslam, et al. 2005, metronidazole, under current guidelines, is the recommended treatment and vancomycin is a second-line agent because of its potential effect on the hospital environment. Gerding et al. 2008 outline the general considerations of the steps to follow for CDI therapy. The initial step is to discontinue treatment with the offending antimicrobial(s) as soon as possible for the patient that is documented or suspected of having CDI. Many patients improve spontaneously with this measure alone (Cascinu, 1995). This strategy was adequate, in the past, for resolution of CDI symptoms in 20%-25% of patients within 48-72 hours but the increased occurrence of fulminant (sudden and severe) CDI and the rapid clinical deterioration of some patients, meant delaying treatment specific for CDI was no longer to be advised, except perhaps for the mildest of illnesses, an antibiotic that is less likely to promote CDI (e. g., a macrolide, sulfamethoxazole, an aminoglycoside, or intravenous vancomycin) may be used instead if it is not possible to stop underlying antimicrobial treatment, though, there are no controlled studies that support this strategy (Gerding et al. 2008)..

Antiperistaltic and opiate agents should be avoided [21]. The severity of disease allows a choice of initial antibiotic therapy for CDI to be made. Treatment of the first or second episodes of CDI are outlined by different regimens, as are recurrent episodes and very severe or fulminant CDI. Also, special circumstances may guide treatment choices, for instance whether or

not the gastrointestinal tract is functioning. It must be clear that intravenous vancomycin is not an option when discussing treatment. Most references to vancomycin therapy involve vancomycin administered orally or via a retention enema. The oral route is preferred for metronidazole therapy unless special circumstances exist.

The First and second episodes.

These drugs in particular have been demonstrated to be efficacious in randomized comparative trails of CDI treatment:

Metronidazole Vancomycin Teicoplanin Fusidic

acid Bacitracin Nitazoxanide Higher-dose tolevamer (which were shown to be

effective in smaller studies of newer potential therapies for CDI). Oral

vancomycin (125 mg 4 times per day for 10- 14 days) and oral

metronidazole (250 mg 4 times per day or 500 mg 3 times per day for 10-14

days) are the agents most studied and with the longest history of use for the

treatment of CDI. Vancomycin is the only treatment approved by the US

Food and Drug Administration (FDA). For several reasons, metronidazole has

historically been used as first-line therapy for most cases of CDI. Oral

vancomycin is more expensive than metronidazole and small, prospective,

randomized trials demonstrated that the 2 agents are equally effective.

Although there is high clinical efficacy, there are a small number of patients

with Clostridium difficile -associated disease (CDAD) do not respond to

treatment with metronidazole (Sanchez et al. 1999). If a patient develops a

second episode of CDI after successful treatment of the first episode,

treatment with the same drug used to treat the first episode is

recommended [33, 39]. A recent study conducted in Canada during an outbreak involving the BI/NAP1 strain of *C. difficile* concluded that first and second episodes of CDI responded similarly, regardless of whether metronidazole or vancomycin was chosen as therapy [39]. However, the investigators noted that complication rates associated with recurrence were greater than previously observed, regardless of which treatment was chosen for the second episode [39]. Extenuating circumstances may exist in some cases, prompting the use of an alternative agent for treatment of a second episode of CDI [39]. For example, vancomycin therapy is preferred if markers for severe CDI are present (such as hypotension or a WBC count of $115,000$ cells/mm³), and alternative routes of therapy should be considered if the functionality of the gastrointestinal tract is affected (as in patients with ileus or toxic megacolon).

Figure 1. Treatment recommendations for the first episode of CDI and for a subsequent episode occurring ≥ 6 months after the initial episode. It is strongly advised that the continued use of non-CDI antibiotic therapy should cease as soon as possible to allow reestablishment of the normal intestinal flora. Pharmacotherapy: Owens RC. Clostridium difficile-associated disease: an emerging threat to patient safety: insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 2006; 26: 299-311.

Figure 2. Treatment recommendations for the third and subsequent episodes of Clostridium difficile infection (CDI) occurring ≥ 6 months after the previous episode. Strongly consider discontinuing non-CDI antibiotic therapy as soon as possible to allow reestablishment of the normal intestinal flora. Pharmacotherapy: Owens RC. Clostridium difficile-associated

disease: an emerging threat to patient safety: insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 2006; 26: 299-311.

Alternative therapies

Treatment with monoclonal antibodies

In a study by Lowry et al. 2010, the natural history of *C. difficile* infection was favourably affected by the administration of fully human monoclonal antibodies when combined with treatment of metronidazole and vancomycin. The rate of recurrent infection among patients treated with standard-of-care antibiotics was reduced as a result of a single infusion of two monoclonal antibodies against *C. difficile* toxins A and B (CDA1 and CDB1). CDA1 and CDB1 are both fully human antibodies that target an exogenous antigen. They observed a significantly reduced recurrence of CDI when they combined the administration of CDA1 and CDB1 with the treatment of antibiotics. Additionally, the administration of a single intravenous dose of antibody may be advantageous, however this does depend on the patient's ability to take oral medications. Further study on this novel treatment needs to be carried out

Immunotherapy

New antimicrobials

Probiotics

Non-toxigenic strains

Toxin binding compounds

Cholestyramine and colestipol are anion exchange resins that reversibly bind toxin and abort the cytotoxic and enterotoxic activity in vitro [52]. In mild diarrhoea without fever or when more conventional therapy fails, cholestyramine (4 g every 6 h) is used because it can bind toxins. However, it cannot eliminate *Clostridium difficile* and it should not be used together with antibiotics because it can also bind them, so reducing their activity (Cascinu)

Vaccines

Faecal transplants

The administration of a stool transplant via a nasogastric tube has been reported anecdotally in the medical literature [14, 15]. This route of administration requires less patient preparation, clinical time, patient inconvenience, and cost than administration of the transplant via a rectal tube or colonoscope. According to a report in a retrospective review by Aas et al. 2003, 15 to 18 patients remained relapse free 90 days post-transplant. They were suffering from recurrent CDI. Lund-Tønnesen, S., Berstad, A., Schreiner A, Midtvedt T.. (1998). *Clostridium difficile*-associated diarrhea treated with homologous feces. *Tidsskr Nor Laegeforen*. 118 (7), 1027-1030. Aas, J., Gessert, C. and Bakken, J. (2003). Refractory *Clostridium difficile*

infection: untraditional treatment of antibiotic-induced colitis. *Clinical Infectious Diseases*. 36 (1), 580–585.

1. 7 Infection control and prevention

Prevention, especially of nosocomial spread, requires isolation and enforced hand washing. Although several studies have investigated bacterial populations in the adult large bowel, in various levels of detail [relatively little information is available concerning the effects of age on these microbiotas. (Hopkins and Macfarlane 2002)]

Chapter 2: Materials and Methods

2. 1. Materials

2. 1. 1. EQUIPMENT

Table 1.

A table or list of equipment and consumables with their location

Equipment Location Personal protective equipment Laboratory Coat Latex gloves (or nitrile gloves if known and proven latex allergy) All staff were issued with Laboratory coats. Latex gloves were available in three sizes, Small, Medium and Large and were kept on each laboratory work bench area. Stocks of latex/nitrile gloves were kept in the pathology store room. Plastic test tubes/ lids On enteric bench. New stock could be found in the Microbiology Main Laboratory stock area. Hettich rotunda 460 Centrifuge

Serology laboratory 4. CP. 21

DS2 Analyser

2. 1. 2. REAGENTS REQUIRED

Table 2.

List of reagents required with their location and COSHH assessment status

Reagent	Location	COSHH Assessment
C. diff Chek tm 60 ELISA	In-use kit in fridge in Microbiology Main Laboratory. Main stock microbiology cold room.	
CAS-MMH-MIC-215	C. difficile TOX A/B II tm toxin ELISA	CAS-MMH-MIC-214

2. 1. 3. QUALITY CONTROL

Negative and positive controls, which were provided in both the C. diff Chek tm - 60 and C. difficile TOX A/B II tm toxin ELISA, were included in every run. A run is valid only if the quality control meets the requirements stated in the kit instructions (Section 3. 1). NEQAS - The laboratory participates in NEQAS where C. difficile is routine specimen type sent as part of the External Quality Assessment Scheme. Discrepant results were reviewed at the weekly microbiology management meetings and reported to laboratory staff at weekly meeting. Any discrepancies arising from either scheme were investigated and a Pathology Non-Conformance and Quality Improvement Form was completed in accordance with the Identification and Control of Nonconformities and Quality Improvement Standard Operating Procedure). Laboratory staff were alerted to the changes instigated following a non-conformance through weekly staff meetings.

2. 2 Method

2. 2. 1 Purpose of examination

The purpose of carrying out these tests was to identify the presence of the C. difficile enzyme GDH and toxin A/B in stool samples using the Techlab EIA kits. Evidence shows that C. difficile enzyme GDH testing has a high sensitivity and negative predictive value and also showed that testing for both C. difficile enzyme GDH and toxin A/B in stool samples as a two-step test is a more accurate method (Shetty et al. 2011).

2. 2. 2 Principle of examination

C. diff Chek tm – 60 glutamate dehydrogenase (GDH) detection kit.

The C. DIFF CHEK - 60 tests used antibodies specific for the glutamate dehydrogenase of C. difficile. The micro-assay plate supplied with the kit contained immobilized polyclonal antibody against the antigen. The conjugate consisted of a highly specific monoclonal antibody conjugated to horseradish peroxidase. In the assay, an aliquot of a faecal specimen was emulsified in the diluent and the diluted specimen was transferred to the microwells containing the conjugate. At this stage, if the antigen was present in the specimen, it would bind to the conjugate and to the immobilized polyclonal antibody during the incubation phase. Any unbound material was removed during the washing steps. Following the addition of substrate, a colour was detected due to the enzyme-antibody-antigen complexes that formed in the presence of antigen.

C. difficile TOX A/B II tm toxin detection kit.

The C. DIFFICILE TOX A/B II test used antibodies to C. difficile toxins A and B. The micro assay wells supplied with the kit contained immobilized affinity-purified polyclonal goat antibody against toxins A and B. The detecting antibody consisted of a mixture of toxin A monoclonal mouse antibody conjugated to horseradish peroxidase and toxin B polyclonal goat antibody conjugated to horseradish peroxidase. In the assay, an aliquot of a faecal specimen was emulsified in the diluent and the diluted specimen was then transferred to the micro well containing the detecting antibody. If toxins A and B were present in the specimen, they would bind to the detecting antibody and to the immobilized polyclonal antibody during the incubation phase. Any unbound material was removed during the washing steps, just as in the C. DIFF CHEK - 60 tests. A colour was detected, following the addition of substrate, due to the enzyme-antibody-antigen complexes that formed in the presence of toxin.

2. 2. 3 Limitations of the procedure

C. diff Chek tm – 60 glutamate dehydrogenase (GDH) detection kit.

A positive result with the C. DIFF CHEK - 60 test DOES NOT confirm the existence of toxigenic strain of C. difficile. Therefore, if a positive result was obtained using the C. DIFF CHEK - 60 test, by suggestion a C. difficile toxin A/B was used to confirm the presence of toxin.

C. difficile TOX A/B II tm toxin detection kit.

Inability to detect toxin A or B in faecal samples from patients suspected of having C. difficile disease may not preclude actual disease but may be caused by other factors (i. e., incorrect specimen collection, handling and/or storage, toxin levels lower than the kit detection limits). The C. DIFFICILE TOX A/B II test would detect Toxin A at levels ≥ 0.8 ng/mL and Toxin B at levels ≥ 2.5 ng/mL. Optimal results with both EIA were obtained with specimens that were less than 24 hours old. Undiluted specimens could be stored between 2° and 8°C for 72 hours before significant degradation of the antigen was noted. If specimens were not assayed within this time period, they would be frozen. Some specimens may have given weak reactions. This may be due to a number of factors such as low levels of GDH antigen or the presence of a weakly toxigenic strain, low levels of toxin production in-vivo, or the presence of binding substances or inactivating enzymes in the faeces.

2. 2. 4. HAZARDS and SAFETY

Containment Level 2 organisms: Caution was taken when making dilutions as there was a chance that there'd be some aerosol production of viable organisms.

2. 2. 5. SAMPLE PREPARATION

Specimen Collection

Specimens for C. difficile were collected in accordance with the Infection control policies and procedure for Management of Clostridium difficile (this was available on the Medway Foundation Trust intranet).

Specimen transport and storage

Specimens were sent in a sterile leak proof container in a sealable plastic bag, transported to pathology reception either by hospital transport system, or by a porter. The transport containers were only be opened in pathology reception area.

Time between specimen collection and processing

Stool specimens were transported to the laboratory within one day. If this was not possible for any reason, the specimen was refrigerated; therefore it was stable for up to 72hrs. During the working week and also at the weekend including Sunday, the specimens were processed for GDH antigen once a day. If any specimens were found to be positive for GDH antigen then the toxin A/B test was carried out before 12. 00hrs the following day. Specimens received into the laboratory before 14. 00 hours on weekdays were processed that same day. Any specimen received after this time was processed the following day. At the weekend, specimens received before 09. 00 hours on Sunday morning were processed that day. These specimens were tested for both the GDH antigen and toxin A/B. All positive GDH antigen specimens from Friday were also confirmed with the toxin A/B test on Sunday unless specifically requested by Consultant Microbiologist or Infection Control.

Sample acceptance

It was a must that the specimen be labelled with sufficient patient identifiers so as to be sure that the specimen unmistakably belonged to that patient, and was required to be accompanied by a completed request form.

Unlabelled, mislabelled or specimens with essential identifiers missing would not get processed in accordance with the Sample Labelling and Rejection criteria. Formed faecal specimens were not suggestive of *C. difficile* disease and therefore were not tested. If a patient specimen was found to be *C. difficile* toxin A/B positive, re-test for a period of 28 days was not necessary.

2. 2. 6. PROCEDURE

The procedures followed in the laboratory are described in the following account.

2. 2. 6. 1 Specimen unpacking

The specimens were brought up to the microbiology main laboratory for unpacking.

2. 2. 6. 2 Specimen labelling

Specimens and request forms were labelled with a faeces section number. Patient details were checked on APEX to establish whether or not the specimen met the Faeces Test Selection.

2. 2. 6. 3 Specimen request entry on APEX

Once the specimen and request form had been numbered they were date stamped. The faeces test selection criteria followed and the APEX test codes were written on the form. The forms were then scanned into DART to facilitate retrieval at a later date and booked onto the APEX system as soon as possible after labelling. Tables 3 and 4 - Apex codes for request entry:

Table 3

Sample type

Apex code

FaecesFTable 4

Sample type

Apex code

C. difficile ScreenGDHC. difficile Toxin A/BCDT

2. 2. 6. 4 Pre-examination processing

The temperatures of the cold room and fridges were monitored daily by the use of temperature charts. If the temperatures were out of range, a Senior BMS was notified so that the appropriate corrective actions could be taken. The C. diff Chek tm - 60 ELISA test kit and C. difficile TOX A/B II tm toxin ELISA test kit were sanctioned to equilibrate to room temperature prior to use. Reagents were dispensed into Sarstedt universal containers (only manufacturer approved by Alere on the DS2) and were clearly labelled with the name of the reagent it contains. When starting on a new kit, old reagents and containers were to be disposed of into a lined autoclave bin and fresh reagents and containers used. Maintenance is performed on the DS2 was ensured daily, weekly and monthly and the maintenance log was completed prior to use. (Use of DS2 is described in Appendix 2) Any problems with the instrument were notified to the Senior BMS.

2. 2. 6. 5 Specimen preparation

One dilution tube was set up for each specimen to be tested. After that, 800µL of diluent was added to each tube and the tube was labelled directly

on the side with a barcode specimen number. Using a plastic pipette, 200µL of faeces specimen was transferred to the sample diluents Vortex well.

Storing the faecal specimen in the diluent was not recommended when testing for the GDH antigen and the sample should have been tested immediately once diluted.

Faecal specimens that needed toxin A/B testing were stored in the diluent at 2°C and 8°C. The specimens were centrifuged using programme 2 on serology centrifuge (3000rpm for 10 minutes). The following consists of lists of step-by-step instructions that were carefully followed

2. 2. 6. 6. Steps for running the C. diff Chek tm – 60 on the DS2.

Turn on instrument (push button located on front, right hand side of instrument). Turn on the PC and printer. The computer should automatically open the Matrix software. Select Normal Mode and OK to operate the DS2 system. The instrument will initialise and a series of self-tests are now performed. A self-test report will be created and shown, and all modules should be shown as 'passed'. If any module is shown as failed inform a Senior BMS. When finished, click the cross in red box to close the report. This allows initialisation to be completed. Click on Tools menu, System configuration and ensure the Sample Tube Type is set to Dynex sample tubes. After selecting the correct tube click OK. Select the number of samples to be tested and click on the Scan Bar Codes box Follow the barcode scan prompts. Select the assay (C. diff Chek 60 GDH) and click Add Assay button. Click Done. The timeline will now appear but is greyed out. Click on

Accept to start the run. Then click Skip. Load samples and consumables as indicated on the screen, clicking the green tick button after each item is loaded. When placing wells into microplate frame, ensure the correct frame is used and that the wells are level and that they are pushed down correctly into the frame. Failure to do so could result in the plate becoming stuck in the instrument and failing to shake. When loading the plate, enter the plate name, lot no, date and initials e. g. GDHlot1345010211AZ. Once all the consumables are loaded, click on the Skip button to start the assay immediately. Once the assay has finished, results can be obtained by clicking on the Report box on the bottom left side of this screen. Then select Print. Perform the end of day maintenance as stated in the use of the DS2. Close down instrument after final use of day.

2. 2. 6. 7. Running the C. difficile TOX A/B II tm on the DS2.

This test is to be performed to confirm all GDH Positive results and should be carried out before 12. 00 hrs. during the working week.

Turn on instrument (push button located on front, right hand side of instrument). Turn on the PC and printer. The computer should automatically open the Matrix software. Select Normal Mode and OK to operate the DS2 system. The instrument will initialise and a series of self-tests are now performed. A self-test report will be created and shown, and all modules should be shown as ' passed'. If any module is shown as failed inform a Senior BMS. When finished, click the cross in red box to close the report. This allows initialisation to be completed. Click on Tools menu, System configuration and ensure the Sample Tube Type is set to Dynex sample

tubes. After selecting the correct tube click OK. Select the number of samples to be tested and click on the Scan Bar Codes box. Follow the barcode scan prompts. Select the assay (C. diff FAST TOX AB II) and click Add Assay button. Click Done. The timeline will now appear but is greyed out. Click on Accept to start the run. Then click Skip. Load samples and consumables as indicated on the screen, clicking the green tick button after each item is loaded. When placing wells into microplate frame, ensure the correct frame is used and that the wells are level and that they are pushed down correctly into the frame. Failure to do so could result in the plate becoming stuck in the instrument and failing to shake. When loading the plate, enter the plate name, lot no, date and initials e. g. CDTlot1345010211AZ. Once all the consumables are loaded, click on the Skip button to start the assay immediately. Once the assay has finished, results can be obtained by clicking on the Report box on the bottom left side of this screen. Then select Print. Perform the end of day maintenance as stated in the use of the DS2. Close down instrument after final use of day.

2. 2. 6. 8. Running the C. diff Chek tm – 60 and the C. difficile TOX A/B II tm on the DS2.

This procedure should only be carried out at the weekend

Turn on instrument (push button located on front, right hand side of instrument). Turn on the PC and printer. The computer should automatically open the Matrix software. Select Normal Mode and OK to operate the DS2 system. The instrument will initialise and a series of self-tests are now performed. A self-test report will be created and shown, and all modules

should be shown as 'passed'. If any module is shown as failed inform a Senior BMS. When finished, click the cross in red box to close the report. This allows initialisation to be completed. Click on Tools menu, System configuration and ensure the Sample Tube Type is set to Dynex sample tubes. After selecting the correct tube click OK. Select the number of samples to be tested and click on the Scan Bar Codes box. Follow the barcode scan prompts. Select the assay (C. diff Chek 60 GDH) and click Add Assay button. Select the assay (C. diff FAST TOX AB II) and click Add Assay button.

NB ALWAYS REQUEST THE C. diff Chek 60 GDH ASSAY BEFORE THE diff FAST TOX AB II.

Click Done. The timeline will now appear but is greyed out. Click on Accept to start the run. Then click Skip. Load samples and consumables as indicated on the screen, clicking the green tick button after each item is loaded. When placing wells into microplate frame, ensure the correct frame is used and that the wells are level and that they are pushed down correctly into the frame. Failure to do so could result in the plate becoming stuck in the instrument and failing to shake. When loading the plate, enter the plate name, lot no, date and initials e. g. (For GDH plate) GDHlot1345010211AZ (For CDT plate) CDTlot1345010211AZ. Once all the consumables are loaded, click on the Skip button to start the assay immediately.

N. B. AS THE ASSAYS ARE PRODUCED BY THE SAME MANUFACTURER THE SUBSTRATE AND STOP REAGENTS ARE THE SAME. The DS2 will only ask you to load these reagents once.

Once the assays have finished, results can be obtained by clicking on the Report box on the bottom left side of this screen. Then select Print. Perform the end of day maintenance as stated in the use of the DS2. Close down instrument after final use of day.

Manual Assay Procedure for C. diff Chek tm – 60 and C. difficile TOX A/B II tm Only use this procedure if the DS2 is Unavailable

Add 200µl of sample diluent to individually labelled dilution tubes. Transfer 50µl of faeces specimen to the diluent. Place the total number of microassay wells required into a microwell plate frame including the controls. Add 1 drop (50µl) of conjugate (red cap) to each well, holding the reagent bottle vertically. Transfer 100µl (2 drops) using the transfer pipette from the accessory kit of each specimen to the assay well. Add 1 drop (50µl) of the positive control (black cap) to the positive control well. Add 50µl of sample diluent (1 drop using pipettes provided) to the negative control well. Cut the adhesive plastic sheet to the size necessary to cover the wells. Cover the wells and incubate them at 37°C ± 2°C for 50 minutes. After incubation carefully shake out the contents of the assay wells into the sink. Wash each well using the prepared wash solution in a squirt bottle with a fine tipped nozzle, directing the Wash Solution to the bottom of each well until full, being careful not to overflow, and then shake the wash solution out into the

sink. Invert the plate onto a dry paper towel. Repeat step 7 a further 4 times using a dry paper towel each time. If any particulate matter is seen in the wells continue washing until it is removed. After washing completely remove any residual liquid in the wells by striking the plate on a dry paper towel until completely dry. Add 2 drops (100µl) of substrate (blue cap) to each well. Gently tap the wells to mix. Incubate at room temperature for 10 minutes. Gently tap the wells at 5 minutes. Add 1 drop (50µl) of stop solution (yellow cap) to each well. Gently tap the wells and wait 2 minutes before reading. Any positive wells should have changed in colour from a blue to a yellow. The plate may be read on the T4 or the DS2 by selecting the read C. difficile reading assay. In the unlikely event of a failure in both these instruments, the plate may be read visually. This concludes the Method and Materials section of this project. Results were collected and recorded and will be outlined in the next section.

Chapter 3: Results & Discussion

3. 1 Results

3. 1. 1 Result interpretation

Results generated for both C. diff Chek tm - 60 and C. difficile TOX A/B II tm ELISA by the DS2 were printed and interpreted according to the assay parameters defined within the kit insert. If the quality control had failed, this would have been indicated on the printout. Plates read on the DS2 or T4 were read at dual wavelength. Visual reading of the microplate was only be carried out if both instruments are unavailable.

Dual wavelength (450/630nm) reading on DS2 or T4

Negative = <0.08 Positive = ≥ 0.08

Visual Reading

Negative = colourless Positive = Definite yellow colour

3. 1. 2 Recording Results

The results generated by the DS2 (or the T4) were printed and the results transcribed to the C. difficile result form ([FRM-MMH-MIC-060] Appendix 1) If a specimen was found to be GDH antigen Positive and it was recorded as POSITIVE in the appropriate column. Then, the specimen then required confirmation by C. difficile toxin A/B and the test was requested on APEX (Section 2. 2. 6. 3). Specimens found negative for GDH were recorded as Negative in the appropriate column. All printout should be attached to the C. difficile result form ([FRM-MMH-MIC-060] Appendix 1) and filed in the C. difficile folder on the enterics bench.

3. 1. 3 Referral of Positive Specimens to Reference Laboratory

There were some specimens that were positive for C. difficile toxin which may have been required to be referred to a reference laboratory for culture and ribotyping by Infection Control. Specimens that required further work (as requested by Infection Control - in outbreak situations) were sent to Southampton HPA for isolation and ribotyping. Infection Control usually provides a list of the maximum number of specimens they would like referred from each outbreak for testing. If and when a suspected outbreak occurred, positive CDT specimens which had been stored at -20°C or lower

were removed from the freezer and an aliquot was sent to Southampton HPA for culture and ribotyping. A report would have been issued with a comment to state that a reference laboratory report would be issued once the culture and ribotype had been identified. Details of the referral were recorded in the other sending away book located in the serology laboratory and the specimens were sent to the following address: Health Protection Agency Collaborating Laboratory DX 6880305 Southampton 90 SO

3. 1. 4 Reporting

3. 1. 4. 1 Urgent Results Procedure

All C. difficile toxin results were considered urgent, were reported as soon as possible after testing. The results were recorded and released on APEX (Section 3. 1. 4. 2 and 3. 1. 4. 3). Infection Control were informed immediately on about all samples tested which included both positive and negative results. All confirmed positive samples were telephoned to the ward to which the patient was admitted. A positive C. difficile telephone record slip should be attached to the original request form and the details should be filled in by the individual that has carried out the tests and informed Infection Control and the Ward.

3. 1. 4. 2 Entering results on Apex

In the test section of the review screen, the appropriate code was entered as follows Table 5 For the C. difficile Screen (GDH) Apex code Full APEX code POS Positive NEG Negative Table 6 For the C. difficile Toxin A/B Apex code Full APEX code DET Detected NDET Not Detected If a patient was found to be C. difficile Screen (GDH) and C. difficile Toxin A/B POSITIVE then the C.

difficile Screen would be suppressed and C. difficile Toxin A/B result would be reported. If a patient was found to be C. difficile Screen (GDH) POSITIVE and C. difficile Toxin A/B NEGATIVE then the C. difficile Screen would be suppressed and C. difficile Toxin A/B result would be reported. This result would be discussed with the Consultant Microbiologist before any report was issued. If further samples were received from these patients, testing of these samples would continue. Specimens that did not meet the criteria for C. difficile testing was reported with the following codes; Table 7 Apex code Full APEX code INS Insufficient sample submitted NT Not Tested When using the Not Tested code an appropriate comment was also added, i. e. Table 8 Apex code Full APEX code FORMED Formed stool samples are not tested for CD Toxin CD1, CD2, CD3 This patient has recently tested positive for C. difficile toxin. A minimum period of four weeks should elapse before retesting for C. difficile. LEAK Specimen leaking on arrival US1, US2, US3 The sample(s) received with this patient's request form were incorrectly or unlabelled. It is unsafe practice to analyse and report results on such samples.

3. 1. 4. 3 Checking and authorising reports

Reports authorisations were carried out in the review section of APEX. Positive results were authorised by Senior BMS staff and negative results by all qualified BMS staff. The details entered on APEX were constantly checked against the details on the request form. If any errors were found then they were amended promptly, prior to any reports being issued.

3. 1. 4. 4 Results from Reference Lab – instructions

Enter reference laboratory result in the review section. The reference laboratory details must be entered in the free text comment prompt. Enter the reference laboratory details in the free text comment box as follows; Press ctrl J Press G At the GET FILE prompt enter SOUTH and press return Then enter 1, 1 and then press return. This expand to; Reference Laboratory Report Number = Reference report from, HPA Southampton Laboratory. Tel 02380 796 408 for Medical Advice The reference laboratory lab numbers were entered in the space provided and the report was authorised as stated in Section 3. 1. 4. 4.

3. 1. 4. 5 Telephoned reports

Authorised results may have been given by MLA and BMS staff. Unauthorised negative results may have been given by qualified BMS staff. Telephoned results from the reference laboratory may be taken by any qualified BMS. The result was supposed to be recorded on APEX, but the reference laboratory report was to be awaited before authorising the report. The telephone log was to be completed by the person giving out or receiving results.

3. 4. 6 Amended Reports

In the event of an incorrect or inaccurate report being issued, an amended additional report needed to be issued. This could only be done by an SBMS, Head BMS. Infection Control was informed as soon as the error was suspected. An IRIS form should have had a completed as well as a Pathology Non-Conformance and Quality Improvement Form.

3. 1. 4. 7 Specimen Retention and Disposal

The specimens were disposed of as stated in the Retention and Disposal of specimens policy.

Discussion

Is where you explain and discuss your findings (results) in the context of the current literature base i. e. your results should be compared with other investigators' work, and any differences / similarities noted. Perhaps your work confirms other peoples' findings - or it may not be the same. Give reasons or suggestions as to why this might be so. Use the discussion to explain your results. The discussion is an important part of your project, and needs to be critical and evaluative – it must analyze and explain your findings in comparison with those of others. Which are more reliable, and why? Did you confirm what others have found (that's fine), or did you find something different (why was this the case?). This should be more than several pages. One paragraph is not sufficient!

Conclusion

Is where you explain the outcomes of your work ' I found X and this means that'... The conclusion is not supposed to be a list of bullet points: you need to explain your conclusions, not list them. Say what have you found out in a concise fashion.

Future Work

should be a fairly short section, indicating (based on your own work) what needs to be done in the future to take the work further. What questions are raised by this work? How would you answer them?

References