Chapter 3

Clostridium difficile Isolation and Culture Techniques

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Abstract

Clostridium difficile infection (CDI) occurs as a disease with a spectrum of severity ranging from mild, self-limiting diarrhoea to a severe colitis, pseudomembraneous colitis or toxic megacolon. The disease arises as a major complication of antibiotic therapy and is most commonly acquired in hospital. The laboratory investigation of faecal samples is supportive of a clinical suspicion that a patient has the disease. Currently the mainstay of diagnosis is the demonstration of *C. difficile* toxins in a diarrhoeal sample; only a few laboratories set up cultures for the organism. However, toxin tests should not be used as stand alone tests since some patients with disease do not have detectable levels of toxin in their faeces. Furthermore, other patients may have large amounts of toxin in the faeces and yet remain well. A combination of tests, therefore, should be used to help the physician to establish a diagnosis of CDI. This combination of tests should include culture (with toxin testing of the isolate), demonstration of toxin direct from the faeces and the detection of *C. difficile* antigen. This chapter outlines the methods used to establish the laboratory diagnosis of CDI and also includes the investigation of environmental samples when it is required to monitor them for the presence of *C. difficile*.

Key words: *Clostridium difficile*, isolation, Cefoxitin Cycloserine Egg Yolk agar, alcohol shock, glutamate dehydrogenase, enterotoxin, cytotoxin.

1. Introduction

Traditionally tests used for the diagnosis of *Clostridium difficile* infection (CDI) have been based on the detection of the organism by culture and demonstration of the toxins and antigen in the faeces of the infected patient.

When *C. difficile* was first described to be a cause of colitis and pseudomembraneous colitis the emphasis was placed on the laboratory's ability to isolate the organism. Selective media were

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developed to separate *C. difficile* from the normal colonic flora. Culture by itself does not differentiate toxigenic from nontoxigenic strains which can both colonise the colon. It soon became apparent that the detection of toxin in the faeces was required to establish that a patient had active CDI. Cytotoxin (toxin B) detection originally used mammalian or primate cell lines (such as HeP2, fibroblasts or Vero cells) to detect the toxin. The toxic effect could then be neutralised with specific antitoxin thereby establishing the specificity of the test.

Enzyme Immunoassays (ELISA) were subsequently developed to give laboratories without technical expertise in tissue culture the ability to detect faecal toxin in a technically easy and rapid manner. The incidence and clinical importance (in terms of morbidity and mortality) of CDI make it imperative that on-site local testing is available in all hospital laboratories.

Many of the commercially available ELISA tests have been clinically evaluated (1, 2). The significance of toxin A negative and toxin B positive (A–B+) strains means that any ELISA kits used must be able to detect both toxins (3). These kits also have a lower sensitivity such that some cases of disease may be missed if only a single faecal toxin test is used. Hence the additional use of so-called toxigenic culture (culture followed by toxin testing of the isolate) has been advocated by some workers (4).

More recently the polymerase chain reaction (PCR) based on the detection of the toxin B gene (*tcdB*) directly from faeces (making it a real-time test) has been stated to enhance the diagnosis of CDI (5, 6). This is likely to become available in a commercial format in the near future.

2. Materials

2.1. Culture and Isolation

1. CCFA (cefoxitin cycloserine fructose agar):
   Dehydrated powder and antibiotic supplement (Oxoid Ltd., Wade Road, Basingstoke, Hants, UK).
2. CCEY (cefoxitin cycloserine egg yolk agar: Brazier's medium):
   Dehydrated powder and the antibiotic supplement (Bioconnections, Thorpe Arch Estate, Wetherby, Leeds, UK).
   CCEY made as pre-poured plates may be purchased commercially (Oxoid Ltd.).
3. CCEYL (cefoxitin cycloserine egg yolk agar with lysozyme):
   Make up as the CCEY formulation and the antibiotic supplement and add a filter sterilised solution of lysozyme to

give a final concentration of 5 mg/l of lysozyme before pouring into Petri-dishes.

4. Egg yolk agar is made by adding egg yolk emulsion (Oxoid Ltd.) to a good anaerobic agar to give a final concentration of 7%.
5. Methylated spirit Industrial 74 OP (Fisher Scientific UK Ltd, Bishop Meadow Rd., Loughborough, Leics, UK).
6. Physiological saline (Oxoid Ltd., UK).
7. Roberston's cooked meat broth (Oxoid Ltd., UK).
8. Phosphate buffered saline tablets (Oxoid Ltd., UK).
9. Ringer's solution tablets (Oxoid Ltd., UK).
10. Spot indole reagent (Bioconnections, UK).
11. *C. difficile* latex kits (Oxoid Ltd., UK).
12. UV light source (360 nm) (Model UVL 21 Black Ray Lamp: UVP Inc., San Gabriel, California, USA).
13. Rodac (contact) plates (Sterilin Ltd., UK).

2.2. Cell Lines and Media

1. HeP2 and other cell lines (e.g. Vero cells, fibroblasts) may be obtained from European Collection of Cell Cultures, CAMR, Salisbury, Wiltshire, SP4 0JG, UK
2. Hanks salt solution; Eagle's MEM plus Earle's salts and glutamine; Foetal Calf Serum, (Sigma-Aldrich Ltd., Fancy Rd., Poole, Dorset, BH12 4QH, UK)
3. Trypsin, penicillin and streptomycin (Sigma-Aldrich Ltd., UK)

3. Methods

3.1. Culture from Faeces

Patients with *C. difficile* infection generally have an inflammatory diarrhoea. Isolation of the organism from the faeces is necessary for epidemiological investigations (including typing) and may be necessary to help for establishing a diagnosis in patients with disease when other tests give equivocal results (*see* **Note 1**) (7).

1. For liquid faeces add an equal volume of faeces and industrial alcohol (*see* **Note 2**) in a sterile container. Mix for 20 s using a vortex mixer to ensure thorough mixing.

   For semisolid faeces emulsify a pea-sized portion of the faeces in physiological saline in a sterile container and mix well. Add an equal volume of industrial alcohol. Mix on a vortex mixer for 20 s to ensure thorough mixing.

2. Leave to stand at room temperature for 30–60 min. Culture approximately 100 μl (three drops from a Pasteur pipette) of the settled faecal layer onto *C. difficile* selective medium (Brazier's CCEY is recommended).
3. Incubate the plates anaerobically at 37°C for 48 h in an anaerobic chamber or anaerobic jar (*see* **Note 3**).

Culture of *C. difficile* from the faeces of patients with diarrhoea followed by toxin testing of the isolate increased the detection rate of diseased patients since some samples from these patients had a negative faecal toxin test (4).

3.2. Culture from Environmental Samples

Because spores of *C. difficile* may suffer prolonged stress whilst in the environment CCEY supplemented with lysozyme (CCEYL) (8, 9) may give better isolation rates from environmental samples (*see* **Note 4**). Environmental sampling may yield useful information when investigating clusters of cases within a unit or ward. It should not be used, however, as a tool for monitoring cleaning efficiency. Routine environmental sampling serves no good purpose.

3.2.1. Water

1. Collect 500 ml samples of water in sterile containers.
2. Filter 100 ml aliquots through 0.45 μm membrane filters.
3. Place the filters into Robertson's cooked meat broth for enrichment and incubate at incubate for 48 h at 37°C.
4. After the 48 h of incubation have elapsed culture the broth onto CCEY or CCEYL and incubate these plates anaerobically at 37°C for 48–72 h.
5. Membranes may be placed directly onto CCEY or CCEYL plates and incubated anaerobically at 37°C for 48–96 h and the colonies counted to give a viable count of cells per 100 ml.

3.2.2. Soil Samples

1. Place 5 g samples of the soil into 5 ml of physiological saline, mix well and add 5 ml of industrial alcohol.
2. Mix on a vortex mixer for 20 s to ensure thorough mixing.
3. Leave to stand at room temperature for 30–60 min.
4. Culture the sediment onto CCEY or CCEYL plates and incubate anaerobically at 37°C for 48 h. Negative cultures may be incubated for a further 48 h before discarding.

3.2.3. Detection of C. difficile from Environmental Surfaces

Surfaces may be sampled by the use of Rodac contact plates or by swabbing surfaces and enriching the organism from these swabs before selective plating (9).

1. Rodac contact plates made using CCEYL medium are pressed firmly against the surface under investigation and held in place for 5–10 s.

2. The plates are incubated anaerobically at 37°C for 48–96 h. Initial examination is made at 48 h and a second further examination after 96 h before discarding. Counts may then be performed of colony forming units (CFU) per unit area if desired.
3. For surfaces that cannot be accessed by contact plates swabbing of surfaces may be performed. Sterile cotton swabs premoistened with nutrient broth are used for swabbing and then placed into a tube of pre-reduced Robertson's cooked meat medium (*see* **Note 5**) incubated anaerobically at 37°C for 48 h.
4. The broth is then subcultured to CCEY and the plates incubated anaerobically at 37°C for 48 h. Negative cultures at this time may be further incubated for another 48 h before discarding as negative.

3.2.4. Detection of C. difficile *in Air Samples*

The recovery of *C. difficile* spores from air occurs in areas where patients with diarrhoea due to *C. difficile* are being nursed. The examination of air samples is not performed routinely for this organism but may be required when complete analysis of ward air is needed.

1. At 30 min intervals (how many samplings are determined by the amount of activity in the identified area) draw 250 l of air through a slit sampler.
2. Concentrate the particular matter into 1 ml of sterile Ringer's solution.
3. Add an equal volume of Industrial alcohol, mix on a vortex mixer for 20 s to ensure thorough mixing and stand at room temperature for 30–60 min.
4. Culture 100 μl aliquots onto CCEYL media and incubate anaerobically at 37°C for 48 h.

3.2.5. Detection of C. difficile *in Meat Samples*

1. Add 5 g of meat sample to 20 ml of enrichment broth (10).
2. Incubate the broth at 37°C for 10 days.
3. After incubation add 2 ml of enrichment broth to 2 ml of industrial alcohol, mix on a vortex mixer for 20 s and stand at room temperature for 30–60 min.
4. Centrifuge this mixture at 3,000×*g* for 10 min.
5. Culture the sediment onto CCEYL agar and incubate anaerobically at 37°C for 48 h. Examine and reincubate the plates for a further 48 h if negative before discarding.

3.3. Differentiation of C. difficile *from Other Similar Clostridia*

On CCEY and CCEYL single well isolated colonies appear as yellow circular colonies with a filamentous edge. One edge of the colony is often pointed toward the direction of the spread. They

are flat in profile with a typical "ground glass" appearance measuring 3–5 mm in diameter. On blood agar incubated for 48 h colonies typically are greyish with a slightly whiter centre, irregular edged and measuring approximately 5 mm in diameter (*see* **Figs. 3.1** and **3.2**).

As CCEY and CCEYL are not completely selective for *C. difficile* a few other clostridia (especially from faecal samples) may grow on these media showing similar colonial morphology

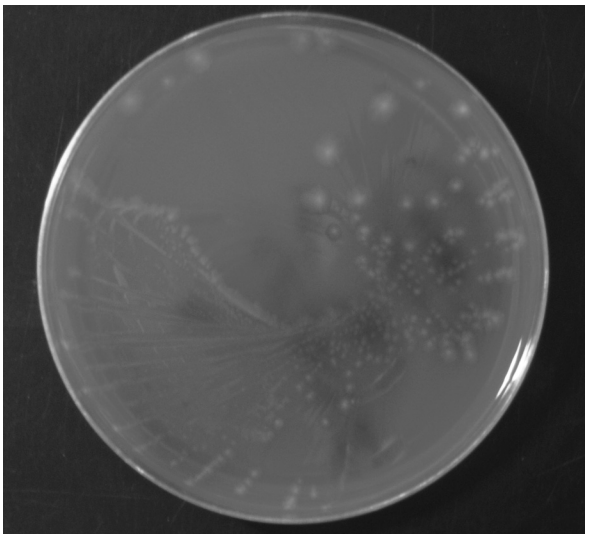

Fig. 3.1. Plate 1 showing *C. difficile* colonies on blood agar; 48 h anaerobic incubation.

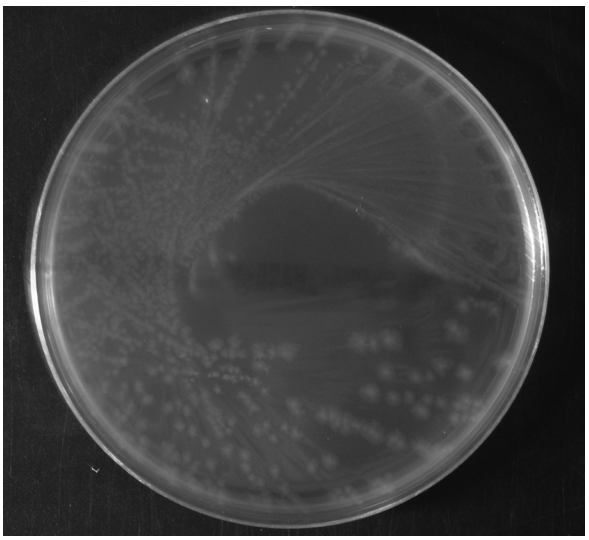

Fig. 3.2. Plate 2 showing *C. difficile* colonies on Brazier's CCEY agar; 48 h anaerobic incubation.

Table 3.1
Biochemical differentiation of organisms capable of growth on CCEY agar

	C. difficile	C. glycolicum	C. innocuum	C. bifermentans *and* C. sordellii
C. difficile latex	+	+	–	+
UV fluorescence[a]	+	–	+	–
Lecithinase on CCEY medium	–	–	–	+
Spot indole	–	–	–	+

[a]Yellow-green.

and may lead to confusion. *Clostridium glycolicum*, *C. innocuum*, *C. bifermentans* and *C. sordellii* may grow on CCEY/CCEYL and therefore, colonies of these species must be differentiated from those of *C. difficile* (**Table 3.1**).

1. Subculture the colonies under investigation onto blood agar to obtain a pure culture.
2. Incubate the culture anaerobically for 48 h at 37°C.
3. Colonies of *C. difficile* possess a typical "elephant dung" or "horse manure" odour.
4. Test the colonies for agglutination with a *C. difficile* latex kit (Oxoid Ltd.).
5. Examine the plate under long-wave UV light (360 nm) to check for yellow-green fluorescence. Wear UV protective goggles or face shield when performing this test.
6. Test the colonies for indole production using the spot indole reagent.
7. Subculture to egg yolk agar and incubate anaerobically at 37°C for 24 h and check for lecithinase production.

These tests should be performed as a minimal panel for the identification of *C. difficile*. Once the isolate is proven to be *C. difficile* the testing the isolate for toxin production may be performed as part of the "toxigenic culture" determination (*see* **Notes 6** and **7** concerning testing isolates for toxin and identification).

3.4. Detection of C. difficile Glutamate Dehydrogenase (GDH) in Faeces

Actively growing vegetative cells of *C. difficile* constitutively produce glutamate dehydrogenase and in patients who are either colonised or infected GDH accumulates in the faeces. This may be detected using a commercial kit which utilises a mouse monoclonal antibody directed against the *C. difficile* specific GDH. Culture and GDH results closely parallel each other (*see* **Note 8**).

The negative predictive value of GDH is very high (NPV = 99.3%) (11) and the absence of GDH from the faeces indicates a very high likelihood that *C. difficile* is not present at the time of testing. All cells of *C. difficile*, both toxigenic and nontoxigenic, produce GDH, therefore, it is not an indicator of potential pathogenicity but may be used as a rapid screening test for the presence/absence of the organism particularly when large numbers of samples are being tested.

1. Make a 1:20 dilution of the liquid faeces in the diluent (to which has been added 1 drop of conjugate). Mix well.
2. Add 400 μl of this mixture to the sample well.
3. Stand at room temperature for 15 min.
4. Add 300 μl of wash buffer to the reaction well. Allow to soak in completely.
5. Add two drops of substrate to the reaction window.
6. Stand at room temperature for a further 10 min and read after the 10 min have elapsed.
7. A positive GDH test is indicated by the appearance of a blue line in the test window.

3.5. Detection of C. difficile *Toxin in Faeces*

Production of toxin by isolates of *C. difficile* occurs in the intestine and is responsible for the inflammatory diarrhoea in susceptible patients. The toxins of *C. difficile* can be detected in the faeces of diseased patients by either using a tissue culture cell line to detect the cytotoxin (toxinB) or by an ELISA for both enterotoxin (toxin A) and cytotoxin (toxin B).

3.5.1. Demonstration of Cytotoxin by Tissue Culture Cell Line (After Edelstein)

3.5.1.1. Preparation of Cell Line

1. Pour off and discard the fluid medium from the master culture in the cell line flask (12).
2. Wash the cells in the flask with Hank's balanced salt solution.
3. Repeat stage 2.
4. Add 5 ml of a 10% solution of trypsin to the washed cells and gently tap the flask making sure the trypsin covers the cell sheet. Stand at room temperature until the cells have detached from the side of the flask.
5. Resuspend the detached cells in 10 ml Earle's MEM medium containing 10% foetal calf serum, 20 units/ml penicillin and 20 μg/ml streptomycin.
6. Perform a cell count using a counting chamber and adjust the cell density to 5×10^4 cells/ml using additional growth medium.
7. Transfer an aliquot to another flask to maintain the continuity of the cell line (if an immortal cell line is being used).

8. Transfer 180 μl of the cell suspension to the number of required wells of a sterile flat-bottomed microtitre tray and cover with the sterile lid.
9. Incubate the tray in 5% carbon dioxide in air at 37°C for 24 h. A monolayer of cells should develop at the bottom of each well.

3.5.1.2. Faecal Preparation

1. Add an equal volume of liquid faeces and phosphate buffered saline (pH 7.0) (PBS) and mix using the vortex mixer for 20 s.
2. Centrifuge this suspension at 3,000×*g* for 15–50 min to obtain a clear supernatant.
3. Filter this clear supernatant through a 0.45 μm membrane filter.
4. Transfer 50 μl of the supernatant into each of two sterile tubes.
5. Add 50 μl of *C. difficile* antitoxin to one tube and 50 μl of PBS to the second tube.

3.5.1.3. Test Proper

Using three wells per analysis add 20 μl of faecal filtrate to well 1, 20 μl of filtrate-antitoxin mixture to well 2 and 20 μl of PBS (negative control) to well 3. If cells in tubes are used add enough stool filtrate to give a 1:10 dilution of the filtrate in the medium in the tube.

1. Inoculate one well in the plate with a filtered (stage 3 above) broth culture supernatant of a known toxigenic strain of *C. difficile* as a positive control to check the performance of the cell line.
2. Incubate the tray in 5–10% carbon dioxide in air at 37°C for 18–24 h and read. Examine using an inverted microscope for any cytopathic effect denoted by rounding of the cells and an increase in their refractility (*see* **Note 9**).
3. Reincubate any negatives for a further 18 h before discarding.

3.5.1.4. Interpretation (Note Re Positives in Wells 1 and 2)

Well 1	Well 2	Well 3	Reported result
Test faecal supernatant well	Test faecal supernatant + *C. difficile* antitoxin	Negative control (PBS only)	
>90% rounding of cells	No change	No change	*C. difficile* toxin detected
No change	No change	No change	No *C. difficile* toxin detected

3.5.1.5. Limitations of the Cytotoxin Assay

1. Tests should be performed on fresh samples. If testing cannot be performed at this time samples may be stored at 4°C until testing can be performed with little reduction in cytotoxin titre (13).
2. Good potency antitoxin should be used. A commercial source of this antitoxin should be sourced by the user (*see* **Note 10**).

3.5.2. Demonstration of Toxins A and B by Commercial ELISA Systems

Laboratories using an ELISA test for the detection of *C. difficile* toxins in faeces almost exclusively use a commercial ELISA kit. These kits are simple to use, convenient and are standardised and controlled by the manufacturer. Meticulous attention to detail is required to ensure optimum performance of the kit (*see* **Note 11**).

It must be remembered that ELISA toxin test results must not be taken as stand alone tests for the diagnosis of *C. difficile* infection as commercial kits do have sensitivity and specificity issues (*see* **Note 12**).

3.5.2.1. Test Principles

1. The ELISA tests are based on the use of antibodies against toxins A and B of *C. difficile*. These antibodies are immobilised on the base of a microtitre tray well or in a line fixed on a pad or membrane.
2. Faecal samples are mixed with a diluent containing a conjugate. The conjugates consist of antibodies to toxin A and toxin B both linked to horseradish peroxidase. These bind any toxin present in the faecal sample.
3. The faecal-conjugate suspension is added to the fixed antibodies (well or membrane) and allowed to react at room temperature for a determined time period during which time any free toxins in the faeces bind to the antibody-conjugate.
4. A washing step or series of washing steps follow to remove any unbound conjugate. A chromogenic substrate (usually tetramethylbenzidine) is then added.
5. After the substrate addition a short room temperature incubation follows to allow the horseradish peroxidase to act on the substrate.
6. When using a plate ELISA a stop solution is then added to develop the colour for reading in a spectrophotometer at 450/620–650 nm. Membrane and pad devices do not require this step as the fixed antibodies appear as a blue line or spot in the device and are read by eye.

3.5.2.2. Limitations to ELISA and Membrane Devices

1. All devices must operate within the manufacturers recommendations giving the desired results with the internal kit controls and the users own external controls.

2. Proper collection of the sample must be ensured (for example, urine must not be present in the faecal sample).
3. A negative test may not exclude the possibility of disease. Some patients with severe disease may demonstrate a negative ELISA test for a number of days (14). The amount of toxin present in the faecal sample may be in a concentration below the limit of detection of the kit being used.
4. Very weak or equivocal reactions should be repeated on a *fresh* specimen submitted from the patient. *Repeating the test on the same sample serves no good purpose.*
5. Some strains of *C. sordellii* may produce toxins that immunologically cross react with the toxins of *C. difficile* (15). However, *C. sordellii* is not thought to cause antibiotic-associated diarrhoea.

3.6. The Future of Detection of C. difficile *in Faecal Samples*

The detection of *C. difficile* in faecal samples using the currently established tests has sensitivity and specificity issues particularly in patients with severe disease. This has led workers in the field to develop real-time PCR methods for the accurate and rapid detection of *C. difficile*. In one prospective multicentre study real-time PCR was compared to an immunocard method and to the cell cytotoxin assay method (used as the gold standard). Based on sensitivity and negative predictive values both the immunocard and the PCR methods were both considered to be useful diagnostic methods (6).

In another study van den Berg et al. concluded that real-time PCR had the highest concordance with toxigenic culture and was the preferred method for the diagnosis of CDI in patients with diarrhoea (16). The use of fluorescence resonance transfer probes in a real-time PCR against toxigenic culture showed the PCR to be sensitive and specific (17). Detection of *C. difficile* in the faeces of patients with CDI using PCR methods will undoubtedly become available commercially in the near future although expense may prohibit the immediate introduction into the diagnostic laboratory.

4. Notes

1. Performing toxigenic culture has been shown to be useful in patients who develop severe CDI but initially have negative tests for toxins both by cell culture assay and by ELISA. Performance of toxigenic culture is given in **Note 6**.
2. Industrial alcohol is methylated spirit (74OP) and can be used in place of absolute ethanol. The methylated spirit is

much cheaper and there is no difference in the ability to select *C. difficile* spores.

3. Cultures may be examined daily without removal from the anaerobic atmosphere when using an anaerobic chamber. When anaerobic jars are used a full 48 h of incubation must elapse before jars are opened. Young colonies of *C. difficile* are particularly sensitive to oxygen on selective agar.
4. The addition of 5 mg/l of lysozyme to CCEY increases the ability to recover spores from environmental samples where smaller numbers of *C. difficile* spores may be present (*see* reference 9). This has been postulated to be due to the ability of lysozyme to overcome the detrimental effect on spore germination that is caused by stress induced damage to cell envelope proteins.
5. Premoistening of swabs with broth increases the ability to pick up organisms and spores from the environmental surface. Robertson's cooked meat medium is a very rich broth medium and gives good growth of organisms from small innocula. Prereduction of the broth is achieved by slightly loosening the cap of the bottle of broth and placing the bottle into a boiling water bath for 20–30 min to drive off any air. After removal from the water bath, tighten the cap of the bottle and allow to cool at room temperature. Use on the same day.
6. Toxin testing of the isolate is done by subculturing a colony into broth and incubating the broth anaerobically at 37°C for 48 h. The broth is centrifuged and the supernatant tested for toxin by ELISA or by cell culture assay (sterilising by filtration through a 0.45 μm filter will be required prior to using tissue culture assay). If this information is urgently required five colonies may be emulsified in the ELISA kit diluent and tested directly. Those that are negative, however, must be confirmed using the broth method.
7. CCEY gives rise to very similar colonies when either *C. glycolicum* or *C. innocuum* is cultured. Definitive identification that the isolate as *C. difficile* is essential to prevent the reporting of a false positive culture and thereby subjecting the patient to unnecessary therapy. The latex reagent reacts with the common antigen which is present in *C. difficile* and strains of *C. glycolicum*, *C. innocuum*, *C. bifermentans* and *C. sordellii*. The other tests are required to prevent false positive reports that may occur if the latex reagent is used as the only test.
8. The author has experience using the GDH commercial kit manufactured by Techlab Inc., Blacksburg, Virginia, USA. It is rare to obtain a positive culture in the presence of a negative GDH test.

9. The appearance of the cytopathic effect of *C. difficile* toxin B may differ depending upon the cell line used. It is recommended that *C. difficile* toxin is used as a control when first setting up the cell line to become familiar with the demonstrated effect.
10. Good potency antitoxin is obtainable from Techlab Inc., USA.
11. Faecal samples should be tested on the day they are taken. *C. difficile* toxins will deteriorate on storage at room temperature. If same day testing cannot be performed the specimen must be kept at 4°C until it can be performed. This must not be more than 72 h. Freezing the specimen may result in loss of toxin titre, especially when samples are repeatedly frozen and thawed (13).
12. This is especially important in patients with severe CDI. The reporting of a negative faecal toxin test in such a patient may result in the denial of effective therapy to a very ill patient allowing the disease to progress.

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