Laboratorium Diagnosis of *Clostridium difficile* Infection

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ABSTRACT

Clostridium difficile is the most important cause of antibiotic associated diarrhea, and pseudomembranous colitis, a severe infection of the colon. Strain Clostridium difficile produce two potent toxin, toxin A (enterotoxin) and toxin B (cytotoxin). These two toxins are both responsible for the diarrhoea and inflammation seen in patients treated due to infection, especially the broad spectrum antibiotics. Direct detection of Clostridium difficile cytotoxin from faecal specimen using mammalian tissue culture lines is considered the standard diagnostics test of Clostridium difficile infection. This test is very sensitive but requires a minimum two days to complete. In order to improve the threshold of diagnosis and treatment, a number of enzyme immunoassay methods have been used, with a reported sensitivity to either toxin A or toxin B.

Keywords: *Clostridium difficile, cytotoxin, diarrhea, enzyme immunoassay*

INTRODUCTION

Clostrodium difficile is ubiquitous in nature and has been isolated from soil, water, and stool of many healthy infant but from the stool from only about 3% of healthy adult volunteers. In a minority of the population, *C. difficile* is a normal intestinal flora, which can be part in as many as 50-80 % of healthy neonatus, and less frequently (3%) in individuals over two years of age. *C. difficile* is a species of bacteria of the genus Clostridium which are gram-positive, anaerobic, spore-forming rods, motile, and characteristic obligate anaerob. The species was named "difficile" because initially it was difficult to culture. Under the microscope after Gram staining, they appear as long drumsticks with a bulge located at their terminal ends (figure 1). *C. difficile* has characteristic antiphagositic polysaccharide capsule. Toxigenic and non toxigenic strains exist.[1-5]

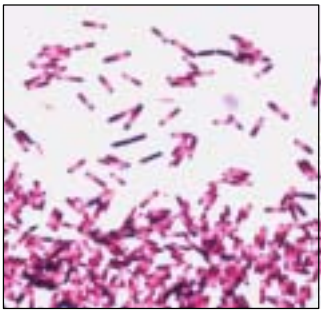

Figure 1. Gram stain of *Clostridium difficile* showing characteristic Gram positive rods[6]

C. difficile virulence are related to fimbriae, flagella and polysaccharide capsule and exotoxin which produced by it, especially the exotoxin. Toxigenic strains of *C. difficile* produce two large toxins: toxin A is a 308 kDa enterotoxin, ad toxin B is a 250/270 kDa cytotoxin. Almost all toxigenic strains posses the genes for, and produce both toxins. Some strains of *C. difficile* are non toxigenic and it is generally accepted that non toxigenic strains are also non pathogenic strains. The strain which recommended has the genes of toxin A and B is VPI 10463.[5,7-11] However, recently, the types of strain which produce toxin B but not toxin A have been described.[11-12] In a recent report from Canada, an outbreak of diarrhoea associated with Toxin A^-B^+ *C. difficile*.[13]

PATHOGENESIS AND PATHOPHYSIOLOGY

In most industrialized country, *C. difficile* is the first organism suspected by health care personal when a hospitalized patient developed diarrhoea. *C. difficile* infection is a nosocomial disease that is spread primarily by medical staff and hospital epidemics are relatively common.[1,14] Most cases of *C. difficile* associated diarrhoea are acquired in hospital although community acquired cases are increasingly being reported.[3]

The precipitating even for *C. difficile* colitis is disruption of the normal colonic microflora. This disruption usually is cost by broad spectrum antibiotic, with clindamycin and broad-spectrum penicillin and cephalosporins most commonly implicated.[4] However, the number of antibiotics nor the duration of therapy was a factor in predisposing patients to infection.[15] The major host factors predisposing patients to the development of symptomatic *C. difficile* infection include advanced age, number and severity of underlying disease, and faulty immune response to *C. difficile* toxins. Increasing rates of community-acquired *C. difficile*-associated infection has also been linked to the use of medication to suppress gastric acid production: H2-receptor antagonists increased the risk twofold, and proton pump inhibitors threefold, mainly in the elderly. It is presumed that increased gastric pH leads to decreased destruction of spores.[4]

Colonisation is thought to result from ingestion of spores, which can survive in extreme environmental condition and persist for months or years. Ingested spores survive the acidity of the stomach and convert to vegetative organism when they reach the colon. The infected patients in hospital are an important reservoir of the organism. Depending on host factors an asymptomatic carrier state or clinical manifestation of *C. difficile* colitis develop.[4,7]

C. difficile toxins binds to specific receptors to stimulate fluid secretion and necrosis of the mucosa associated with an inflammatory infiltrate.[15] Toxin A causes fluid secretion and mucosal damage, resulting in diarrhoea and inflammation. Toxin B is a powerful cytotoxin, 1000 times more potent in tissue cultures than toxin A. Both toxin activate the release of cytokines of human monocyte, and this effect may be responsible for the colonic inflammation seen in pseudomembranous colitis.[5,9,16] The clinical presentation ranges from mild diarrhoea to life-threatening disease, such as fulminant pseudo-membranous colitis and toxic megacolon (figure 2).[3,4]

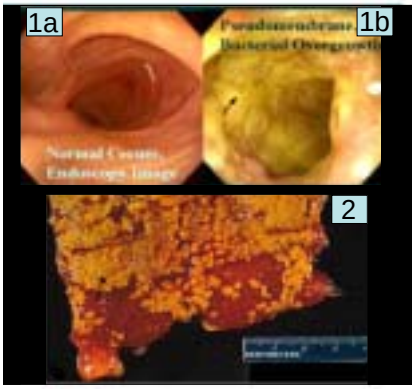

Figure 2. 1a: normal cecum, 1b & 2: cecum with pseudo-membrane[17]

COLLECTION AND TRANSPORT OF SPECIMENS CONTAINING *C. DIFFICILE*

The diagnosis is usually based on the analysis of fresh diarrhoeic stool. The minimal volume of fresh stool is about 5 to 50 millilitres or 5 to 50 grams.[2] Well-formed stools and samples from patients younger than one year of age should be rejected in the laboratory unless an epidemiologic study of stool carriage is being conducted.[3,18] Other suitable specimens include biopsy material or lumen contents obtained by colonoscopy and involved bowel. The samples have to be put in capped plastic container for transport of the specimens and should be promptly sent to the laboratory. The samples should be examined in two hours, or could be stored in 4°C or freeze. Toxin denaturation occurred if samples remain at room temperature longer than two hours.[2,8,11] However, for shipment of specimens to a reference laboratory for a toxin assay, we recommend they be shipped on dry ice. On the other hand, an anaerobic transport container (transported at 25°C) should be used for specimens to be processed for isolation and identification of *C. difficile*. Specimens to be processed for the latex agglutination test should not be frozen, because the antigen detected is unstable on freezing.[2]

DIAGNOSIS LABORATORY

The laboratory method for the diagnosis of *C. difficile* infection are essentially detection of the organism and demonstration of its toxins.[7] The assay to detect the organism are culture and Gram stain, common antigen detection using immunoassay method (IA) or latex agglutination test, and polymerase chain reaction (PCR) method. Immunoassay for detection of *C. difficile* toxins sometimes combine with common antigen detection, have become available. Rapid staining techniques, such as Gram stain or fluorescent antibodies are not recommended.[3,19]

The laboratory diagnosis of *C. difficile* infection depends on toxin detection in diarrhoeal stool. It is generally accepted that non toxigenic strains are also non pathogenic strains.[7] The gold standard test for *C. difficile* infection is stool/tissue culture assay for toxin B (CBA). In this test cell cultures are used to demonstrate cytotoxicity in the presence of stool extract, which is prevented by administration of antitoxin. This test has excellent sensitivity and specificity, and can detect toxin B concentrations as low as 10 pg/mL.[3,18-22]

Cytotoxin B assay detect the presence of toxin B in a cellular culture using cell line such as human lung fibroblast (MRC-5), Chinese hamster ovary (CHO), Vero, or Hep2. The standard procedure CBA is, faecal specimens were prepared and according to each manufacturer's instruction and centrifuged.

The supernatant was collected, then filtered through a 0.2 ìm pore size filter. Then inoculate the filtrate into cell monolayer and incubate within 24-48 hours. The principle of CBA is characteristic cytopathic effect (CPE) neutralized by antitoxin was interpreted as a positive result.[3,4,11,18] However, it is also the least controlled test, and non specific reaction is common in some laboratories in the hands of an inexperienced technologist. The addition of too much faecal material to the tissue culture well can cause false positive reactions.[1] Toxin B is a heat-labile protein; therefore, samples must be refrigerated immediately upon collection. The CBA take 24 to 48 hours to complete, and some hospitals may not have an in-house tissue culture facility, which can delay diagnosis. CBA test could be perform on the sample of culture colony, called "cytotoxin culture assay". This method can increase the sensitivity up to 99% with the longer time consuming (72-92 hours) and more expensive.[3,18-19,23]

Recently, the most common laboratory test for diagnosis *C. difficile*-mediated disease is an enzyme immunoassay that detects toxin A, the combination of toxin A and B, or the common antigen in one kit.[1,4] This test provide quick result and less expensive too. These systems have a good specificity, but their sensitivity is low, as they cannot detect quantities of toxin below 100-1000 pg. Therefore, there is a false negative rate of 10 - 20 %.[3,18] Those that detect both toxin A and toxin B are preferred than detect only toxin A. For A^-B^+ strains are capable of spreading and causing outbreaks and fatal disease, the recommended assay is the test that can detect both toxin.[1,3] The principle of procedure of EIA is, during the first incubation, *C. difficile* toxin A + B present in stool supernatant are captured by antibodies attached to the wells. The second incubation adds additional antitoxin A + B antibodies that sandwiches the antigen. The next incubation adds an anti-second antibody conjugated to peroxidase. After washing to remove unbound enzyme, a chromogen is added which develops a blue colour in the presence of the enzyme complex and peroxide. The stop solution ends the reaction and turns the blue colour to yellow.[21,,24] The Infectious Diseases Society of America and The Society for Hospital Epidemiology of America have device guidelines for detecting *C. difficile* toxin (table 1).[18]

Table 1. Guidelines for the use of the *Clostridium difficile* toxin assay[18]

Only diarrheal stools should be tested unless there is ileus
"A test of cure" should not be performed except as part of an epidemiologic investigation
Only specimens from patients who are older than one year of age should be tested
Enzyme immunoassay is an acceptable alternative to the cytotoxin assay but is less sensitive
Diarrhea that develops after three days of hospitalization should be tested only for *C. difficile* toxin (the three-days rule)

C. difficile can be isolated by the use of spore selection technique (i.e. heat shock or alcohol selection procedures) and by use of selective plating media containing phenyl ethyl alcohol (PEA) blood agar, or cycloserine-cefoxitin, egg yolk and fructose agar (CCFA), and colonies may be identified by characteristic fluorescence. Presumptive colonies were characteristic by a yellowish colour, flat morphology, yellow-green fluorescence, and a horsey smell. CCFA plates were incubated anaerobically at 37°C for a minimum two days. In addition to spore selection procedure, a plate of PEA or CCFA medium should be inoculate with untreated stool or stool suspension prepared in buffered gelatine diluent. However, culture is not specific for pathogenic toxin-producing *C. difficile* strains and, therefore is not clinically helpful except for strain typing in outbreaks of nosocomial infection as well as epidemiologic and clinical research.[2,4,11,21,25-26]

Some commercial test for common antigen of *C. difficile*, specific glutamate dehydrogenase (GDH) has been available for more than 10 years. The initial kit on the market was latex agglutination test, but a lateral device with coloured latex conjugate and flowthrough tests with enzyme conjugates have become available. GDH is an essential enzyme and is produced constitutively by all *C. difficile* isolates. Like bacterial culture, test that detect common antigen do not distinguish toxin-producing from non-toxigenic isolates. Even so, the common antigen has proven to be a good screening marker for *C. difficile* because the enzyme is produced in large amounts and can readily be detected in faecal specimens. The commercial GDH tests offer a turn-around time of 15 to 45 minutes, which is another reason the test are used in laboratory. The greatest utility of the common antigen tests is their use as a screen to rule specimens negative and to select specimens for further testing, a toxin test. A combination immunoassay for GDH and toxin A and toxin B have become available with a high sensitivity (97%) and specificity (97 to 99%). The advantages of this test are same day turn-around and high negative predictive value.[1,4,19]

The tissue damage to the intestinal mucosa by toxins A and B leads to a rapid influx of inflammatory cells. The inflammatory response plays a key role in how quickly the disease progress es to colitis and whether the disease develops into pseudomembranous colitis, which is life-threatening if left untreated. Levels of faecal lactoferrin, which is released from the secondary granules of faecal leukocytes and other inflammatory markers rise significantly in patients with advanced *C. difficile* disease compared with levels in patients with milder case of the disease. Thus, the presence of elevated faecal lactoferrin may help

Table 2. Characteristic of the main diagnostics for *Clostridium difficile* infection[3]

Methods	Detects	Pros	Cons
Culture	Microorganism	High sensitivity	Low specificity for detection of toxigenic strains Requires 48 h
Cytotoxicity from the sample	Toxin B	Sensitivity	Sophisticated equipment Requires 48 h
Cytotoxicity from the culture	Toxin B	High sensitivity	Sophisticated equipment Requires 72-92 h
Enzyme immunoassay test	Toxin A or A + B	Specific and rapid	Low sensitivity
Polymerase chain reaction	Different targets	High sensitivity and specificity	Costly and time-consuming technique

to define the severity of the disease. Tests that monitor the level of intestinal inflammation will provide valuable information to physicians, alerting them to the need for prompt therapy with metronidazole and vancomycin when there is inflammation in the presence of the organism.[1,27]

The Polymerase Chain Reaction (PCR) has been used to detect *C. difficile* in stool specimens and to identify toxigenic strains among isolates, and directly from primary culture plates, by using primers based on the genes for toxin A and toxin B. These method are highly specific and sensitive, but further work on the direct detection on *C. difficile* and its toxin in stool samples is needed before they can be used routinely.[7] Simon et al[28] report the first real time PCR assay for detection of *C. difficile*. It is a rapid, sensitive, and specific and allows detection of *C. difficile* directly from stool samples.

There are some characteristic of the main diagnostics techniques for *C. difficile* infection (table 2).

CONCLUSION

Toxin A (enterotoxin) and toxin B (cytotoxin) of *Clostridium difficile* are both responsible for the diarrhoea and inflammation in patients treated especially with the broad spectrum antibiotics. Direct detection of *C. difficile* cytotoxin from faecal specimen using mammalian tissue culture lines is considered the standard diagnostics test of *C. difficile* infection. However, it is also the least controlled test, and non specific reaction is common in some laboratories in the hands of an inexperienced technologist and some hospitals may not have an in-house tissue culture facility, which can delay diagnosis.

In order to improve the threshold of diagnosis and treatment, a number of Elisa immunoassay method have been used. The most common laboratory test for diagnosis *C. difficile*-mediated disease is an enzyme immunoassay that detects toxin A, the combination of toxin A and B, or the common antigen in one kit. This test provides fast result, less expensive, and has a good specificity, although their sensitivity is relatively low.

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