



## BRITISH COLUMBIA ASSOCIATION OF MEDICAL MICROBIOLOGISTS (BCAMM)

### Laboratory Diagnosis of *Clostridium difficile* Associated Disease

#### Introductory Comments

*Clostridium difficile* is recognized to be the major cause of bacterial diarrhea acquired in the adult health care setting<sup>1</sup>, and is recently reported to cause diarrhea in healthy persons living in the community and in peripartum women<sup>2</sup>. The spectrum of illness in patients with *C. difficile*-associated disease (CDAD) ranges from mild watery diarrhea to severe bloody diarrhea, abdominal cramping and pseudomembranous colitis, which can be fatal. The major risk factor for development of CDAD is preceding antibiotic therapy<sup>3</sup>, although anti-neoplastic agents<sup>4</sup> and proton-pump inhibitors<sup>5</sup> have also been implicated. In some cases no inciting agents have been identified<sup>2</sup>. Clindamycin and cephalosporins have been the antibiotics most commonly associated with CDAD, but recent increase in the incidence of disease implicates fluoroquinolones<sup>6,7</sup>. Treatment may be as simple as removing the inciting antibiotic if possible, but often requires oral antibiotic therapy, and the disease is characterized by frequent relapses requiring additional antibiotic or alternative therapies.

*C. difficile* colonization of the gastrointestinal tract may be found in approximately 3% of healthy adults and 20-40% of hospitalized patients<sup>8,9</sup>, although the colonization frequency of toxin-producing isolates is unknown. In infants, rates of colonization and toxin detection as high as 65% have been reported. After the first year of age, *C. difficile* colonization decreases significantly, and by age 2 asymptomatic carriage is similar to that of adults<sup>10,11</sup>.

*C. difficile* is an anaerobic gram positive spore forming organism, and presumably antibiotic therapy disrupts the balance of normal flora allowing the spores to germinate and become metabolically active. The clinical manifestations of CDAD are due to the toxins produced by the vegetative form. Two toxins have been long-recognized and well-described, toxin A (an enterotoxin), and toxin B (a cytotoxin)<sup>12</sup>. Recent outbreaks in several centers in North America including Montreal and surrounding regions in Quebec have noted an increase in the incidence and severity of CDAD<sup>13,14</sup>. Studies of strains associated with these outbreaks have revealed the emergence of a new strain which has genetic changes leading to the production of 16 to 23 times more toxins A and B in vitro and a new toxin named binary toxin<sup>7</sup>. The emergence of these new strains has emphasized the importance of the laboratory diagnosis of CDAD.

Laboratory confirmation of the clinical suspicion of CDAD is complicated by the presence of the bacteria in normal adults, and by the toxin-mediated nature of the disease. Simple stool culture for to detect the presence of *C. difficile* does not alone make the diagnosis, as the presence of toxins, often labile, must be confirmed. There are many tests available for the confirmation of CDAD, and several different approaches which can be used to maximize the sensitivity and specificity of laboratory detection. This document will review the laboratory diagnosis of CDAD, highlighting specimen collection and transport, the available diagnostic tests, and provide several different diagnostic approaches for consideration, with the advantages and disadvantages of each.

## **C. difficile Associated Disease – Clinical Aspects**

### **Case Definition**<sup>15</sup>

- acute onset of diarrhea (more than three loose stools within a 24 hour period) for 2 days (48 hours) without another etiology. Loose stool is defined as that which takes the shape of the container that holds it.
- **AND one or more of the following:**
  - laboratory confirmation (positive toxin or culture with evidence of toxin production)
  - or**
  - diagnosis of typical pseudomembranes on sigmoidoscopy or colonoscopy or histological/pathological diagnosis of CDAD
  - or**
  - diagnosis of toxic megacolon

### **Risk Factors for CDAD**<sup>16, 17, 18</sup>

- history of prior antibiotic use
  - may occur days to months after the antibiotic use, or more remote antimicrobial use
  - may present after a single dose of antibiotics
  - nearly every antimicrobial class has been reported to be associated with CDAD, the most common being cephalosporins, clindamycin, broad-spectrum penicillins, fluoroquinolones, and ampicillin
- proton pump inhibitor use
- gastrointestinal tract procedures/surgery
- medications which alter intestinal motility
- cancer chemotherapy
- advanced age
- hospitalization for >72 hours
- no other known etiology of the diarrhea

### **Clinical Presentation**<sup>16, 17, 18</sup>

- presentation ranges from asymptomatic to fulminant, life-threatening colitis
- mild to moderate usually non-bloody watery diarrhea with abdominal cramping
- pseudomembranous colitis: systemic signs and symptoms such as fever, more frequent diarrhea, leukocytosis, abdominal pain/cramping, abdominal tenderness, thickening of the bowel wall on imaging studies, presence of adherent white/yellow coloured pseudomembranes on sigmoidoscopy or colonoscopy
- toxic megacolon: dilatation of the large intestine with severe systemic symptoms which may lead to bowel perforation

### **Treatment**

- in mild cases (<5 bowel movement per day) withdrawing the inciting agent may resolve symptoms in up to 10% of patients
- specific antimicrobial therapy directed towards *C. difficile*
- up to 25% reported relapse rate even with specific antimicrobial therapy
- consult authoritative references for specific therapy guidelines

## **Special Pediatric Considerations**

Young infants are often colonized with toxin-producing *C. difficile* (up to 65%) but have no symptoms from the organism or its toxins for reasons that are still unclear, possibly relating to a lack of receptors for the toxins in this age group<sup>19</sup>. Testing performed on stool from infants less than 12 months of age who develop diarrhea after taking antibiotics have a high probability of getting a positive result even though *C. difficile* is unrelated to the infants' illness. A positive result is more likely to cause a problem (complication due to unnecessary treatment) than cure it. However, severe or fatal *C. difficile* disease has been reported to occur in infants with Hirschsprung disease or in severely neutropenic children with leukemia<sup>20</sup>.

The following approach is recommended in children less than 24 months old:

1. Testing in children younger than 12 months of age should not be performed, except in some rare exceptions, ie. infants with Hirschsprung's disease or severely neutropenic children with haematological malignancies. It is recommended to communicate to the physician the reason for specimen rejection by adding a comment on the report, such as:  
"Please note: Specimen not processed. *Clostridium difficile* toxin testing is not performed on stool specimens of children  $\leq 12$  months of age as up to 65% of healthy infants have asymptomatic carriage of this organism. Other causes of diarrhea should be considered."
2. Testing in children between 12 and 24 months of age is discouraged due to the lack of specificity of the testing. An interpretative comment should be included in the report if the testing is done and found positive, such as:  
"Asymptomatic carriage of *Clostridium difficile* toxin may occur in children < 2 years of age. Clinical correlation is required."

## **Specimen Collection and Transport**

**Note:** Testing **should not** be performed on asymptomatic patients.

1. Collect stool specimen in sterile dry container without transport medium or preservative.
2. Transport the specimen to the laboratory promptly.
3. Refrigerate specimen if not processed within 24 hours of collection. Toxins degrade at room temperature.

Note: Some kit manufacturers recommend freezing specimens if processing is delayed; therefore, it is recommended to refer to the instructions provided by the manufacturer.

## **Rejection Criteria**

1. Reject specimens that are not soft or liquid. Specimens should take the shape of container.
2. Rectal swabs are unacceptable as they do not provide enough material for testing.
3. Laboratory will request repeat collection if there is not enough specimen for testing unless the specimen is from patient with ileus or obtained at endoscopy.
4. Patients with positive tests should *not* have repeat testing, including test of cure, unless they have relapse of their symptoms after completion of therapy. Toxins may be detectable for weeks to months after therapy.
5. Symptomatic patients suspected to have CDAD with negative stool toxin assay should have repeat testing, preferably by another method. Sensitivity of most EIA methods is not high enough to exclude CDAD on a single negative toxin result. Specimen can be referred for more specialized testing such as cell culture cytotoxin assay or toxigenic culture.

## Laboratory Diagnosis of *C. difficile* Associated Disease

### Overview

There are two basic approaches to the laboratory diagnosis of *C. difficile* associated disease. Both depend on testing of stool specimens.

- Detection of the organism itself: *C. difficile* culture with subsequent toxin assessment (toxigenic *C. difficile* culture), antigen detection of the organism (often combined with toxin assay in commercial systems), or nucleic acid amplification (including detection of toxin genes).
- Detection of the toxins produced by the organism: EIA, cell culture.

### Methods for Detection of the Organism

#### **Toxigenic *C. difficile* culture**

- Stool specimens are inoculated to selective/differential media that foster growth and detection of the organism amid the normal bowel flora. Preferred media are CCFA (cycloserine-cefoxitin-fructose-egg yolk agar) or Difficile agar (cycloserine-cefoxitin sheep blood agar).
- Potential *C. difficile* isolates must be identified and tested for toxin genes by nucleic acid amplification or for toxin production by cell culture assay of culture broths.
- Performance:
  - Turn-around-time (TAT): 48-72h
  - Complexity: high
  - Cost: moderate
  - Toxigenic *C. difficile* culture has the highest sensitivity (>95%) and equivalent specificity (>99%) compared to other methods for the laboratory diagnosis of *C. difficile* infection<sup>21</sup>.
  - Toxigenic culture provides isolates for epidemiological typing and/or virulence testing.

#### ***C. difficile* common antigen detection**

- Detection of *C. difficile* common antigen, subsequently identified as glutamate dehydrogenase, is offered in commercially available EIA test kits. A latex agglutination version of the test is also available, but experience with this test has been variable, and the test is rarely used.
- Performance:
  - TAT: rapid
  - Complexity: low
  - Cost: high
  - Antigen EIA: sensitivity 84-89%; specificity 90-98%<sup>22, 23, 24</sup>.
  - This test cannot distinguish between toxigenic and non-toxigenic *C. difficile* (see example of potential application below).

## **Nucleic acid amplification**

- Direct detection of *C. difficile* in stool specimens by nucleic acid amplification has been described, but problems of sensitivity and reliable nucleic acid extraction from stool specimens limits its potential for clinical application.

## **Methods for Toxin Detection**

### **Cell culture cytotoxicity**

- Stool specimens are centrifuged and bacteria are removed from the supernatant by membrane filtration.
- Fibroblast monolayers are exposed to the sterile stool filtrates with and without neutralizing antibody.
- The monolayers are observed for cytotoxicity at 48 and 72h.
- Typical cytopathic effect neutralized by specific antibody is a positive test.
- Performance:
  - TAT: 24-48h
  - Complexity: moderate to high
  - Cost: moderate
  - Sensitivity 80-85%; specificity 99%.

### **Toxin EIA**

- Direct stool EIA tests for toxin A and/or B.
- Toxin A negative strains occur, so current recommendations are to test for both toxins.
- Many commercial kits available.
- Performance:
  - TAT: rapid
  - Complexity: moderate
  - Cost: moderate
  - Sensitivity 50-70%; specificity 98-99%

## **Approaches to the Laboratory Confirmation of CDAD**

- There is no universally accepted approach to the laboratory diagnosis of *C. difficile* associated disease.
- Three possible approaches and an assessment of their benefits and limitations are listed below.

### **(1) Direct Detection of Toxin A/B in Stool Specimens**

- Any of a variety of commercial EIA kits for toxins A and B, or cell culture cytotoxicity for toxin B, can be used to detect *C. difficile* toxin in stool specimens.
- Assessment:
  - Toxin A/B EIA provides rapid, but low sensitivity results; cell culture assay for toxin B provides improved sensitivity but slow turn-around-time.
  - A single negative toxin result does not rule out *C. difficile* disease.
  - Toxin negative patients suspected to have *C. difficile* infection should have repeat testing preferably by another method such as toxigenic culture.

## **(2) Antigen plus Toxin Detection in Stool Specimens by EIA**

- Combined Toxin A/C. *difficile* antigen EIA kit or separate kits for antigen EIA and Toxin A/B EIA used together.
- Assessment:
  - Combined toxin and antigen testing provides rapid results and good sensitivity, but low specificity due to detection of both toxigenic and non-toxigenic *C. difficile* by the antigen detection assay.
  - Toxin EIA negative/antigen positive patients should be retested by an alternate method such as cell culture cytotoxicity or toxigenic culture.

## **(3) Direct Toxin A/B Stool EIA plus Toxigenic Culture by PCR**

- Combined testing by toxin A/B EIA and *C. difficile* culture with PCR based confirmation of toxigenicity.
- Assessment:
  - Complementary testing methods that offer both rapid turn-around-time (EIA) and high sensitivity (toxigenic culture) for detection of *C. difficile* associated disease.
  - The approach recommended by the Society for Healthcare Epidemiology of America<sup>25</sup>.

## **(4) Other**

- Combinations of complementary testing methods improve the accuracy of CDAD detection.
- Other combined approaches, such as cell culture cytotoxicity plus toxigenic culture, may be worthy of consideration.

## Public Health / Epidemiology Aspects

### Epidemiology of CDAD in Canada

Recent reports in the literature have suggested an increase in incidence, severity, and relapse rates of CDAD in Canada<sup>26</sup>. Since the last half of 2002, several hospitals in Quebec have experienced a dramatic increase in serious disease associated with CDAD. Similar reports have been released in the US and UK<sup>27, 28</sup>.

In 1997 CNISP (the Canadian Nosocomial Infection Surveillance Program) conducted a six week prospective surveillance study with 19 acute care hospitals across Canada. Two of the participating hospitals were located in BC. The mean number of nosocomial CDAD cases was 5.9/1000 patient admissions. The crude mortality rate was 15.2%, with 1.5% of deaths directly or indirectly related to CDAD<sup>28</sup>.

From November 2004 to April 2005, CNISP again undertook a prospective surveillance project. The definition for CDAD used is the same as that proposed in this document. Each participating hospital used their current procedures to identify *C. difficile* in patient stools. In addition, all specimens were frozen and transported to NML (National Microbiology Laboratory) in Winnipeg, Manitoba. At NML strains were isolated from the specimens and subjected to PFGE fingerprinting, antimicrobial susceptibility, and PCR to identify toxigenic strains (toxin A&B), plus a test for the presence of binary toxin and deletion of the regulatory gene *Tcd C* gene<sup>29</sup>.

Results of the study showed a mean number of cases as 4.3/1000 patient admissions with a range from 0.3 to 13.6. 14.9 % of patients died, 2.3% of deaths were directly related to CDAD and 3.5% of deaths were indirectly related. Of the 525 strains fingerprinted, 23% were the same hyper toxin producing strain known to cause higher death rates in the US and UK.

Compared to the rest of the country, Ontario and Quebec have 2.3 more cases of CDAD. The mean number of CDAD/ 1000 patient admissions for the western provinces was 2.4. The number of nosocomial CDAD has decreased across Canada as a whole, but the number of deaths directly or indirectly related to CDAD has increased 4-fold as compared to the six month time period measured in 1997. The hyper toxin producing strain that has been shown to cause outbreaks in Quebec was also identified in hospitals across Canada, including BC. No outbreaks involving this clone have been identified to date in BC.

### BCCDC Public Health and Reference Testing

As the provincial public health reference laboratory, BCCDC Laboratory Services offers the following CDAD services:

1. *C. difficile* toxin testing is available as part of the investigation of gastrointestinal disease outbreaks or clusters provided by the BCCDC (Outbreak Coordinator, Environmental Services Laboratory). This outbreak testing is carried out as part of a cascade of tests (GI Outbreak Test Algorithm), should clinical presentation and the initial algorithm testing indicate and specimens are negative for Norovirus by RT-PCR. Clinical

information from outbreaks and use of this algorithm approach may be discussed with the Outbreak Coordinator or the Medical Microbiologist on-call.

2. BCCDC Laboratory Services will also provide further testing for confirmation of suspected outbreaks or clusters of CDAD in health care facilities. Requests for these investigations can be made through the Infection Control Consultant during regular business hours. After hours, weekends, and holidays, requests for these investigations can be made through the Medical Microbiologist on-call.
3. Testing available at BCCDC includes:
  - Toxin assay by cell culture cytotoxicity.
  - Culture for *C. difficile*.
  - Pulsed Field Gel Electrophoresis (PFGE) testing. All isolates from an outbreak will be tested for relatedness by PFGE according to the NML protocol. The DNA gel fragment profile for each isolate will be defined by PFGE patterns archived in the NML *C. difficile* PFGE database. BCCDC has been certified in this testing by NML.
  - For strains that are suspected of being hyper toxin producers (PFGE pattern similarity to known hyper toxin producing strains), an analysis of the *Tcd C* gene genotype will be performed. Current known hyper toxin producing strains harbor 18 or 39 base pair in-frame deletions in the *Tcd C* gene. A nonsense *Tcd C* mutant has also been reported. The *Tcd C* gene product is a putative negative regulator for the *C. difficile* toxin A and B genes<sup>30</sup>.
  - A review of the usefulness in a public health setting of an immunological screen assay used primarily for outbreak investigation is underway.

Since testing services are multi-laboratory at BCCDC, co-ordination of tests must be done through either the BCCDC Outbreak Coordinator (604-660-6079), the Infection Control Consultant (604-660-6076/ pgr. 604-632-9394), or the Medical Microbiologist on-call (604-661-7033).

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