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Newer Diagnostic Methods in *Clostridium difficile* Infection

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Abstract

Clostridium difficile infection (CDI) is a public health problem causing severe morbidity and mortality. The clinical presentations of CDI vary from asymptomatic carriage to the full blown pseudo membranous colitis (PMC). With the arrival of the hyper virulent NAP1/BI/027 *C. difficile* strain, increased incidence of more severe clinical conditions inclusive of PMC, toxic megacolon and intestinal perforation are being reported from the West. Additionally, the recognition of community-acquired CDI signals the presence of several risk factors. Accurate diagnosis of CDI is essential for ongoing epidemiology, optimal treatment and prevention but continues to be challenging. During the past 30 years no standard laboratory test for CDI diagnosis has been clearly established. Diagnostic approaches for CDI are based on several aspects. Clinically the signs and symptoms are watery or bloody diarrhea, abdominal cramps, fever, leukocytosis, etc. PMC can be diagnosed endoscopically as multiple yellow-white friable plaques, a few centimeters in size, attached to the underlying mucosa. Computed tomography scan findings does not help diagnosis, but may help in initiating specific therapy against CDI. Culture can be used for epidemiological and antibiogram purposes during outbreaks. Tissue cultures, enzyme immunoassays and molecular assays are useful to detect *C. difficile* toxins. Glutamate dehydrogenase test helps to screen out a large number of samples. Toxigenic culture is based on the isolation of *C. difficile* in culture and then detecting its toxigenic status. The implications of a false negative or a false positive test can lead to disastrous consequence. There are currently two reference assays for the diagnosis of CDI with different targets: the cytotoxicity assay that detects free toxins and the toxigenic culture which detects the organism with the potential to produce toxin. CDI diagnostic testing is an important issue and clinical laboratory professionals should use the assays which give the best performance for the detection of CDI.

Keywords: *Clostridium difficile*; CDI; Diagnostic approaches; Toxigenic culture

Introduction

Clostridium difficile infection (CDI) is a growing nosocomial and public health problem with mortality up to 25% in frail elderly people. The majority of hospitalized patients infected by *C. difficile* are asymptomatic carriers, who serve as silent reservoirs for continued *C. difficile* contamination of the hospital environment. The clinical presentations of CDI in increasing order of severity include asymptomatic carriage, colitis without pseudo-membrane formation, pseudo membranous colitis (PMC) and fulminant colitis with catastrophic transmural inflammation and myonecrosis. However the most severe forms are the least common. Surgical patients comprise 55-75% of all patients with CDI due to the fact that perioperative prophylaxis requires the use of antibiotics. The arrival of a mutant hypervirulent *C. difficile* bacterial strain, NAP1/BI/027 (North American PFGE type I/ restriction endonuclease analysis BI/ribotype 027) with 16-23 times higher levels of toxin production has increased the incidence of more severe clinical conditions like PMC, toxic megacolon and intestinal perforation. Additionally, the recognition of community-acquired CDI signals the presence of several risk factors. Approximately 15-20% of CDI patients relapse after successful treatment with the standard antibiotics of choice *i.e.* vancomycin or metronidazole [1] usually within a week of stopping the treatment. The small bowel and the appendix may also act as reservoirs of *C. difficile* spores that enter the colon and result in relapse. Detection of *C. difficile* in clinical specimens may not always be associated with disease and therefore the diagnosis of CDI continues to be a challenge for both laboratories and clinicians [2].

Accurate diagnosis of CDI is essential for ongoing epidemiology, optimal treatment and prevention but continues to be challenging. The present article is an overview of the current state of CDI diagnosis and discusses the strengths and limitations of laboratory tests based on available literature.

Diagnostic Methods for CDI

Diagnosis of CDI is based on several approaches and the most relevant ones are detailed below:

Diagnosis based on clinical signs and symptoms

Clinically, the disease can be diagnosed by symptoms of profuse, watery, green, foul-smelling or bloody diarrhea accompanied by abdominal cramps. No leukocytosis is seen when benign diarrhea occurs with antibiotic use. However in severely ill patients white blood cell counts of 20,000 per mL or greater may be found. At times, patients may have occult colonic bleeding, and occasionally may develop copious hematochezia. Other common manifestations include high fever, nausea, anorexia, malaise, dehydration and delirium. Hypoalbuminemia of 3.0 g/dL or lower may be observed in severely ill patients [3] with ascites as the only presenting expression of PMC. Patients with ileus may have minimal diarrhea resulting in accumulation of secretions in the dilated, atonic colon. Patients, who relapse once, increase their chances for further relapses with the same or different strains of *C. difficile*. Clinical suspicion for CDI is

therefore important because stool assays for diagnosing CDI are not widely available and if available it is laden with inherent problems thereby delaying or missing the diagnosis.

Endoscopy

Fulminant colitis may occur in about three percent of CDI patients and may account for most of the serious complications including perforation, prolonged ileus, megacolon and death [4]. By inserting a flexible endoscope with a camera, the colon can be examined. PMC is the classic presentation of a full-blown case of CDI. The manifestation appears later in the disease and therefore may not be present always. PMC when present appears as multiple yellow-white friable plaques, a few centimeters in size, attached to the underlying mucosa. About 10% cases of PMC go undetected when only sigmoidoscopy is done as the distribution of the membrane is patchy and occurs in the proximal colon when it begins. Other features that may also be present are edema, blurring of the vascular pattern and thickening and blunting of the haustral folds. Once PMC has been established there is no need of a biopsy unless confirmation of CDI is required. Biopsy is confirmatory but not essential, unless the mucosa appears inflamed, friable, granular or hemorrhagic and PMC has not been detected to reveal histologic changes typical of PMC. The delay in the diagnosis of PMC could be lethal due to development of toxic megacolon (>7 cm diameter) accompanied by severe systemic toxicity or perforation. Endoscopy should be avoided in patients with paralytic ileus or colonic dilatation because of the risk of perforation. It is better reserved for special situations, such as when the patient is seriously ill and the results of rapid but not highly sensitive non-invasive tests are negative or delayed and CDI is strongly suspected. However, at times other disorders may also produce similar pseudo membranes.

Computed tomographic scan/abdominal X-rays

PMC can sometimes be diagnosed by computed tomographic (CT) scan when diarrhea is absent but abdominal pain, fever and leukocytosis occurs. Barium enema examination should be avoided because of the risk of perforation and precipitation of megacolon. CT scan findings do not diagnose PMC, but may actually help in initiating of specific therapy for CDI. They are most useful in PMC cases localized to the proximal colon and may reveal colonic distension, thickening, pericolonic inflammation, or free air. The patient may also have dilated small intestine with air-fluid levels mimicking intestinal obstruction or ischemia or pseudo-obstruction, or even a perforation. Abdominal plain films may also demonstrate small bowel dilatation, air-fluid levels (mimicking an intestinal obstruction or ischemia), and "thumb printing" (scalloping of the bowel wall) due to submucosal edema.

Conventional culture

Clostridium difficile grows on selective media providing a low cost method. The media generally contain antibiotics (cycloserin and cefoxitin) to ensure selectivity, and sometimes taurocholate or lysozyme to promote germination of spores and to enhance the sensitivity of the media. Chromogenic media are now available and allow an easy and more rapid identification of *C. difficile* due to the black color of the colonies. Ethanol or heat shock can be performed before plating in order to reduce the endogenous flora and optimize the recovery of *C. difficile* strains. Sensitivity is approximately 2000 bacteria/g of stool. But culture is dependent upon the presence of spores or viable vegetative cells. The procedure is cumbersome and requires several days for results. Moreover it requires a follow-up toxin testing as only about a third of the colonized isolates produce toxin. Culture is however useful for epidemiological and antibiogram studies particularly during CDI outbreaks.

Tissue culture

C. difficile toxins can be detected in the fecal samples of CDI patients using tissue culture assay which has been regarded as the gold standard. Different cell lines can be used, but McCoy, MRC-5 and Vero are considered to be the most sensitive. It can detect as little as 1.0 pg of toxin B. The disadvantages of tissue culture method are the difficulty in maintenance of cell cultures and the procedure being expensive and time-consuming. False negative results can occur in stored samples due to toxin degradation or by delay in transportation of the samples or by medication administered to the patient. In fact, a negative cytotoxicity assay does not completely rule out *C. difficile* as the cause of diarrhea as 30% of patients may be missed [5].

Enzyme immunoassays

Enzyme immunoassay (EIA) to detect toxin A or both toxins A and B in stool samples is widely used the world over. It has sensitivity up to 90% and specificity up to 100%. But the common EIAs have been reported to have sensitivity values less than 50% [6]. The advantage of EIA is predominantly the speed with which results are obtained, roughly two and a half hours. But the high cost per single test may necessitate batching of samples. During unavoidable circumstances, stool specimens can be tested even if unrefrigerated for up to 13 h after collection [7] but only in countries with cold climate.

Glutamate dehydrogenase (GDH) test (TechLab, Blacksburg, VA) is as good as culture in sensitivity. The GDH component of the *C. DIFF* Quik Chek Complete test and both PCR methods were highly sensitive for the detection of toxigenic *C. difficile* organisms in stool specimens [8]. These tests can therefore be relied upon for the exclusion of *C. difficile* carriage or infection and are ideally suited to screening large number of specimens, as the results are quickly available [9].

Toxigenic culture

Toxigenic culture is also considered a gold standard for CDI diagnosis and has been approved by US Food and Drug Administration (US FDA). This is a two-step method based on the isolation of *C. difficile* in culture. The capacity of the strain to produce toxins *in vitro* is then determined by CTA (by inoculating colonies in broth and testing the supernatant on cell culture) or by EIAs for toxins A and B performed directly on colonies (this latter application is not always validated by the manufacturers). PCR targeting *tcdA* and/or *tcdB* after DNA extraction from colonies can also be performed in order to detect the presence of the genes encoding for toxins.

Molecular techniques

PCR to detect toxin A or toxin B genes has sensitivity similar to cytotoxin testing. Peterson *et al* [6] investigated ten different diagnostic assays for CDI of which only the US Food and Drug Administration–cleared qPCR assay (Becton Dickinson, Franklin Lakes, NJ) and 1 glutamate dehydrogenase test (TechLab, Blacksburg, VA) were not statistically inferior to culture in sensitivity. This sensitive molecular test can rapidly detect the *C. difficile* toxin B gene in stool samples and is highly accurate. It is now being adapted by

several laboratories and becoming more widely available. Real-time PCR tests that detect toxin A and B genes are highly sensitive and specific. The sensitivity of PCR is greater than EIA and comparable to cytotoxicity assay. In addition, PCR results can be available within as little as one hour. As PCR will detect even low number of *C. difficile* organisms also present in healthy individuals, it may give rise to wrong CDI diagnosis. Given its high sensitivity and potential for false positive results, PCR can be used in an algorithm together with other assays such as EIA for GDH and EIA for toxins A and B. The PCR methods offer greater specificity, although their cost is greater and there is a risk that mutations in the toxin B gene may reduce their sensitivity in the future, which may go undetected if PCR is used alone.

A real-time cell analysis assay (ACEA Biosciences, CA, USA) based on electronic impedance technology was described in 2010 for quantitative detection of toxin B in stool samples. The system provides automated data acquisition in real-time and is amenable to a high-throughput, on-demand platform. However, this test is not routinely used in clinical laboratories.

In 2009, nucleic acid amplification tests (NAATs) based on the detection of toxin genes became commercially available for the diagnosis of CDI [10]. These methods have been compared to toxigenic culture (equivalent endpoint) and showed a good correlation. Results can be provided to clinicians within the same day of the receipt of the stool sample. According to the different assays, the tests are amenable to both batch and on-demand testing. The cost of these assays is still prohibitive for many laboratories and their place among the different diagnostic options remains to be clarified. In particular, these tests again raise the crucial question of the clinical significance of the presence of a toxigenic strain without any free toxin in stools.

Adjunct to CDI diagnosis

Both the toxins induce mucosal injury and colitis as seen by neutrophil infiltration, which is a prominent feature of CDI. Intestinal inflammation can be evaluated in fecal samples by lactoferrin [11] and myeloperoxidase assays [12]. Myeloperoxidase enzyme is released from the primary granules whereas lactoferrin is released from the secondary granules of polymorphonuclear cells and both of these rises significantly in patients with advanced CDI. Thus MPO and fecal lactoferrin assays are good biomarker for inflammation in inflammatory diseases and may be used as a quantitative index of inflammation. These assays performed simultaneously with *C. difficile* toxin assay can help rule out asymptomatic carriage of *C. difficile* [13] (Table 1).

Sr. No	Methods	Strengths	Limitations
1	Clinical diagnosis	Clinical suspicion important as stool assays not widely available.	Patients with ileus may have minimal diarrhea making clinical suspicion difficult.
2	Endoscopy	PMC can be detected and biopsy can be taken.	Manifestation appears late. May not be present always; risk of perforation in paralytic ileus and colonic dilatation.
3	Computed tomographic scan/abdominal x-rays	May reveal colonic distension, thickening, pericolonic inflammation etc.	Risk of perforation and precipitation of megacolon by barium enema examination.
4	Conventional culture	Low cost method; useful for epidemiological and antibiogram purposes.	Dependent upon spores/viable vegetative cells. Procedure cumbersome and time consuming. Requires follow-up toxin testing.
5	Tissue culture	Gold standard for toxin detection.	Maintenance of cell cultures difficult; procedure expensive and time-consuming. False negative results with stored samples.
6	Enzyme immunoassays	Rapid results obtained. GDH test good for screening large numbers.	Low sensitivity with most EIAs.
7	Toxigenic culture	Gold standard for CDI diagnosis.	Organism has to be cultured for toxin production. Cumbersome.
8	Molecular techniques	Detect toxin A and B genes. Sensitivity greater than EIA. Rapid results obtained.	Cost prohibitive. Clinical significance of toxin gene presence without free toxin in stools questionable.

Table 1: Comparison of Different Diagnostic Methods.

Implications of False Negative and False Positive Test Results

The implications of a false negative test result can lead to (i) inappropriate medical management (ii) worsening CDI due to continued non-*C. difficile* specific antibiotic treatment (iii) lack of specific treatment for CDI (iv) spread of *C. difficile* to other patients due to lack of isolation and consequently leading to potential outbreaks (v) under-reporting and inaccurate epidemiological data (vi) spread of *C. difficile* to other institutions when the patient changes place of treatment, and (vii) negative economic consequence to hospitals. In case of a false positive test result, inappropriately discontinued antibiotic for original disease can (i) worsen the infection as well as the diarrhea because of lack of appropriate treatment (ii)

miss diagnosis of the actual cause of diarrhea (iii) lead to over-reporting and inaccurate epidemiology data (iv) lead to unnecessary patient isolation as well as possible compromised medical care and (v) bring anxiety to the patient and the family.

Recommendations

The best standard laboratory test for diagnosis has not been clearly established for the past 30 years. There are currently two reference assays for the diagnosis of CDI with different targets: the cytotoxicity assay that detects free toxins and the toxigenic culture which detects the organism with the potential to produce toxin. Only stools from patients with diarrhea should be tested for *C. difficile.*

Nucleic acid amplification tests for *C. difficile* toxin genes such as PCR are superior to EIA for toxins A+B testing as a standard diagnostic test for CDI.

GDH screening tests for *C difficile* can be used with subsequent toxin A and B EIA testing, but the sensitivity is lower than NAATs.

Repeat stool testing for *C. difficile* toxins should be discouraged as subsequent test do not affect medical management or isolation procedure.

Testing for cure of CDI should not be done because most patients with positive toxin at the end of therapy do not relapse.

Conclusion

Testing of stool from patients without clinical indications of *C. difficile* diarrhea is an unnecessary expense. It will simply complicate patient care if unnecessary antibiotic treatment is given to such a patient. Testing for CDI should be based on the age of the patient, length of hospital stay, the presence of clinically significant diarrhea precipitated by antibiotic or other drug intake, the presence of acute abdominal syndrome with little or no diarrhea and underlying co-morbidities. CDI diagnostic testing is an important issue and clinical laboratory professionals should use the assays which give the best performance for the detection of CDI.

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