Review Article

Laboratory diagnosis of *Clostridium difficile* infection

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Abstract: *Clostridium difficile* infection (CDI) is a serious and sometimes life-threatening illness caused by toxin release from *Clostridium difficile* (CD), a gram-positive anaerobic bacterium. Infection with CD can cause clinical manifestations in a spectrum from asymptomatic carrier states to pseudomembranous colitis and toxic megacolon. Accurate diagnosis of CDI depends on early recognition of clinical symptoms of diarrhea, fever, and cramps especially after antibiotic use. Bacterial culture can be performed for epidemiological and antibiogram purposes during outbreaks of CDI. Culture, enzyme immunoassays (EIA), and molecular assays are useful for diagnosis of CDI. Toxigenic culture is useful to determine the cytopathic effect of the bacteria. Current Infectious Disease Society of America (IDSA) and American College of Gastroenterology (ACG) guidelines recommend using nucleic acid amplification tests or glutamate dehydrogenase (GDH) antigen followed by EIA testing for CD toxin A and B. Future studies for CDI diagnosis are looking toward toxin identification and the use of metabolomic analysis.

Keywords: *Clostridium difficile* (CD); laboratory diagnosis; culture; assay

Received: 23 January 2018; Accepted: 05 February 2018; Published: 03 March 2018.
doi: 10.21037/jlpm.2018.02.05
View this article at: http://dx.doi.org/10.21037/jlpm.2018.02.05

Introduction

Clostridium difficile (CD) is a gram-positive, anaerobic, rod-shaped bacterium that exists in either a vegetative or spore form. The bacterium can be traced back to the early 1900s and, in Europe and North America, is now one of the leading causes of the hospital-acquired infections in the US (1). The clinical effects of CD infection (CDI) range from mild diarrhea to fulminant colitis leading to death. In 2007, data from U.S. death certificates revealed that CDI was associated with approximately 14,000 deaths, making CDI a leading cause of gastroenteritis-associated deaths in the U.S. (2). Hospitalizations for CDI have doubled since the year 2000, and are projected to increase (3). This is in part due to rampant use of broad-spectrum antibiotics and aging population with multiple comorbid illnesses. In 2015, the estimated healthcare cost related to CDI was approximately $4.8 billion (2).

The primary risk factor for development of CDI is the antibiotic use. Antibiotics are known to disrupt the normal gut flora, which is protective against colonic colonization of CD (4). Antibiotics associated with high incidence of colitogenic (tending to cause colitis) potential include clindamycin, quinolones, cephalosporins, and amoxicillin/clavulanic acid (5,6). Two toxins, toxin A (TcdA) and toxin B (TcdB) are the primary virulence factors that contribute to pathogenesis of CDI.

Pathogenesis

After the disruption of the normal colonic flora, toxigenic bacterial strains proliferate and release toxin (1,7). It is currently believed that only toxigenic forms cause disease in humans (1,8). TcdA and TcdB are the most commonly reported toxins associated with CDI. All known toxigenic strains contain TcdB (1,9). TcdA and TcdB are encoded on a pathogenicity locus, which contains genes encoding for positive and negative regulators of toxin expression and a holin (small cytolytic proteins in the bacterium's cytoplasmic membrane that contributes to formation of "holes" in the host's cell wall) thought to promote release of the toxins from the organism (9). These toxins act by

disrupting the actin cytoskeleton on fibroblasts in tissue culture cells by glycosylating the small GTPases Rho, Rac, and Cdc42 (9,10). The glycosylation prevents the activation of these proteins and their ability to regulate actin polymerization, leading to cell death and stimulation of an inflammatory cascade resulting in the symptoms of diarrhea and pseudomembranous colitis in CDI (1,9,10). It is important to differentiate toxigenic from non-toxigenic stains of CD. Different stages of the development of CDI will have either the organism present, detectable free toxin, or both. This process has important potential implications for diagnostic testing (7).

Various methods of laboratory diagnosis of CDI

Bacterial and toxin culture

CD culture can be performed with conventional bacterial culture, tissue culture, or toxigenic culture (11). Conventional culture requires selective media for germination of spores isolated from a stool specimen (11,12). Two notable agars are cycloserine-cefoxitin-fructose-agar (CCFA) and ChromID *C. difficile* agar (12). CCFA is a commonly used medium, and the colonies produced are grayish or yellow-green, if seen under ultraviolet light, and tend to smell like manure. Gram staining reveals the typically described gram-positive rod shape organism. ChromID *C. difficile* agar produces black colonies, which, if allowed to incubate for 48 hours, have a sensitivity of up to 100% (12). In multiple studies, ChromID *C. difficile* agar provided greater sensitivity than CCFA, even when the latter agar was enriched with sodium taurocholate, egg yolk, or tryptone soy agar with sheep blood (12). Bacterial cultures are time consuming requiring several days for results and require follow-up toxin testing (11).

Tissue culture assays use a tissue culture format to detect cytotoxicity or cytopathic effect (CPE) in stool samples as well as confirm the presence of bacterial toxin with the use of antitoxin (13). An example of this is Cell Cytotoxicity Neutralization Assay (CCNA), which detects CD toxins in fecal samples with human cell monolayers (14). The fecal sample is inoculated onto sensitive tissue culture cells and allowed to incubate for at least 48 hours (15). Human and animal cell lines such as McCoy, MRC-5, and Vero are commonly used and are considered the most sensitive (11,12). If toxin is present, the cells round up into a CPE. The CPE for isolated cell lines is then re-evaluated with antitoxin (15). If antitoxin reverses the CPE, the test is positive (12,15). CCNA is the standard test for toxins in stool. Tissue culture assay is difficult due to cell maintenance. Tissue culture is also time-consuming and expensive (11).

Toxigenic culture is a time-consuming (greater than 72 hours) two-step method, primarily used for reference or as part of a diagnostic algorithm (12,16). CD is cultured, colonies are tested for toxin production, and the CPE or the toxigenic status is evaluated and neutralized by an antitoxin (5,12).

Occasionally culture is essential for evaluation of antibiotic resistance and ribotype (a molecular technique that utilizes the unique make up of DNA or rRNA to identify and classify bacteria) testing (5). When the culture is combined with CCNA or polymerase chain reaction (PCR), toxigenic culture is considered to be the gold standard test for CDI diagnosis (11,17). Evidence suggests that patients with positive toxigenic culture and positive cytotoxic assay have a worse outcome than those with a negative CCNA result.

Glutamate dehydrogenase (GDH) assay

GDH enables the bacteria to manage oxidative stress by inactivating hydrogen peroxide (12). The GDH assay is considered a reliable screening tool for CDI (5,12). This is supported by a 2016 systemic review and meta-analysis, which found the GDH assay had high sensitivity and specificity as well as low cost, thus is considered an appropriate screening test (18). The test is recommended as an initial test in a multi-step algorithm recommended as per the ACG guidelines (6). GDH antigen is produced by both toxigenic and non-toxigenic strains of CD. If a GDH assay is positive, samples must undergo a confirmatory testing with enzyme immunoassay (EIA) or by molecular testing for toxigenic infection (5,12). CCNA and toxigenic culture are not usually used because of they are time-consuming and expensive (12). The sensitivity of the GDH assay is as high as that of bacterial culture (11), and it is inexpensive, thereby providing an economical method to quickly "rule out" CDI (12).

EIA for toxin detection

EIA was one of the initial methods for CDI detection (12). Today, EIA is widely used for detection of TdcA alone or simultaneous detection of both TdcA and TdcB in fecal samples (12). EIA is beneficial for CD testing because of its

lower cost and results can be obtained within hours (2 to 2.5 hours). However, the results of EIA are often inconsistent due to its poor analytical sensitivity (12). Sensitivity of EIA ranges from values less than 50% to 90% (11). The range of sensitivity of EIA are related to numerous factors such as antigenic variation of toxins of different strains, inadequate handling of samples (including storage and transportation), and laboratory technical variance (12). To avoid false positive results, EIA is mainly used in multi-step algorithm for CD diagnosis (6,11,12).

Molecular tests

Nucleic acid amplification tests (NAATs) detect pathogen-specific DNA or RNA sequences (12). NAAT assays utilize either PCR or loop-mediated isothermal amplification (LAMP) to target CD toxin genes (17). These tests are particularly important due to their ability to provide rapid results, with high sensitivity and specificity (5,12). Numerous NAATs are currently available. The Xpert CD assay is an example of a NAAT utilizing multiplex PCR (enhances multiple targets during a single PCR) (19). Xpert CD assay was studied recently in a 2017 meta-analysis and re-enforced the benefits of NAATs due to their high sensitivity and specificity as well as rapid turn-around time (19). The disadvantages of these tests include higher cost due to the need for trained personnel, high false positive rates, and detection of non-toxigenic strains (20). To reduce false positives, NAAT can be used as part of a diagnostic algorithm. Due to the rapidity and high accuracy, NAAT are currently adapted by several laboratories and is widely available for CD testing (11). The inability of the test to detect biologically active toxin in fecal samples remains a shortcoming to be addressed, since it is believed that toxins expressed by CD are the organism's main virulence factor, and their presence is necessary for pathogenicity and diagnosis of CDI (21).

Additional testing with biomarkers

Inflammation induced by toxins from CD can be evaluated in fecal samples using lactoferrin, myeloperoxidase, cytokines, and calprotectin (11,12). Lactoferrin and calprotectin are both present in inflammatory bowel disease (IBD) and intestinal inflammatory processes. Lactoferrin is a normal component of milk and is present in noninfectious IBD (6). These laboratory biomarkers, though easy to perform but are not specific, but they have potential to be uses as the indicators of disease severity (12). However, the testing for these laboratory biomarkers is not recommended by any major society because of lack of specificity and clinical evidence (6).

Guideline recommendations

IDSA and ACG guidelines recommend testing for CD only if stools are diarrheal and take the shape of the container. International CDI guidelines recommend a multi-step approach combining an initial sensitive test with a confirmatory test for CDI diagnosis (5). Both the ACG and the IDSA guidelines recommend NAAT for CD toxin genes, as a standard diagnostic test for CDI which is superior to EIA for detection of toxins A + B. GDH screening tests for CD can be used in two- or three-step screening algorithms with subsequent toxin A and B EIA testing, but the sensitivity of such strategies is lower than NAATs (6,22). Presence of pseudomembranous colitis upon endoscopy (flexible sigmoidoscopy and colonoscopy) can also diagnose CDI, but is not currently recommended due to friable colonic mucosa causing increased risk of colonic rupture (5). None of the major gastrointestinal and infectious disease societies recommend repeat testing for CD (5,6,12).

New developments

The one of the areas of exploration for CD testing has focused on the detection of the bacteria's toxins. Ultrasensitive and quantitative digital enzyme-linked immunosorbent assays (ELISA) are currently being developed that allow detection and quantification of both TdcA and TdcB (23). The digital ELISA is the first assay to rapidly detect both toxins produced by CD and is the first use of single molecule array (Simoa) technology, which allows labeling of single fecal protein molecules (23).

Another avenue of upcoming research involves metabolomic analysis of stool and urine. Metabolomic research takes advantage of the gut microbiome disturbance that occurs in CDI due to antibiotic use (24,25). Studies involving the metabolomic analysis of the stool microbiome have discovered that stool samples of CDI subjects *vs.* health controls have altered levels of fecal cholesterol and coprostanol (24). Urine-based metabolomic analysis, from a 2016 pilot study, found significant differences between CDI groups and healthy controls (25). Out of the 53 metabolites used in the study, choline was found to be the single most

important metabolite to differentiate controls from CDI patients (25).

Conclusions

In conclusion, CDI is a major public health concern. Rapid recognition and diagnosis of CDI is essential, and a wide variety of tests have been developed to meet diagnostic needs. Unfortunately, no single rapid, accurate test exists for the diagnosis of CDI. Current IDSA and ACG guidelines recommend NAATs or multi-step testing with GHD assays. Research development of CD testing is using continued exploration of the toxins produced by the bacteria as well as metabolomic analysis of stool and urine.

Acknowledgments

Special thanks to Dr. Allen Tindol for assistance with editing.
Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editor (Hemant Goyal) for the series "Role of biomarkers in gastrointestinal disorders" published in *Journal of Laboratory and Precision Medicine*. The article has undergone external peer review.

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jlpm.2018.02.05). The series "Role of biomarkers in gastrointestinal disorders" was commissioned by the editorial office without any funding or sponsorship. The author has no other conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/jlpm.2018.02.05
Cite this article as: Kendrick K. Laboratory diagnosis of *Clostridium difficile* infection. J Lab Precis Med 2018;3:20.

