Effective Utilization of Evolving Methods for the Laboratory Diagnosis of *Clostridium difficile* Infection

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Physicians should understand the performance characteristics of evolving laboratory tests used to diagnose *Clostridium difficile* infection if they are to correctly integrate test results with clinical information and formulate an appropriate therapeutic intervention for patients with antibiotic-associated diarrhea.

*Clostridium difficile* is an important emerging nosocomial pathogen [1]. Individuals may be asymptomatically colonized by *C. difficile*, and the rate of carriage is higher in hospitalized patients than in the general population [2, 3]. Clinical presentation of active *C. difficile* infection (CDI) occurs in susceptible individuals who are unable to mount a sufficient anamnestic immune response and ranges from mild diarrhea to severe, debilitating complications [4]. Antimicrobial therapy frequently precedes *C. difficile* gastrointestinal infection and presumably contributes to onset by altering the balance of the intestinal flora [4]. Historically, clindamycin, cephhalosporins, and amoxicillin were most frequently associated with CDI [5]. More recently, the increased use of fluoroquinolones has propelled this class to the top of the list of CDI-associated antimicrobials [6].

In the United States, CDI accounts for 15%–25% of nosocomial antibiotic-associated diarrhea and is the leading cause of infectious antibiotic-associated diarrhea [7, 8]. Billions of health care dollars are spent on CDI treatment annually, and costs are expected to escalate, because the number of cases and the severity of disease are steadily increasing [9, 10]. The emergence of the highly virulent BI/NAP1/027 (toxinotype III) strain accounts for most but not all of this increase in frequency and severity and has contributed to increasing mortality rates [9, 11, 12]. There has been a disproportionate increase in incidence and mortality in patients >65 years of age, and progressively more cases of community-acquired (vs nosocomial) disease have been documented. Importantly, community-acquired cases of CDI are less likely to be associated with antecedent antibiotic exposure than are nosocomial cases [5, 13]. Rapid and accurate diagnosis of CDI for treatment and prevention is crucial in this setting of increased risk, increased incidence of infection, and increased morbidity and mortality (6%–15%) [7].

**BACTERIOLOGY**

*C. difficile* is an anaerobic gram-positive, spore-forming, toxin-producing bacillus that was first described in 1935 as a component of the intestinal flora in healthy newborns. Its name reflects the difficulty encountered by investigators who attempted to isolate and grow these *Clostridia* on conventional media. Phenotypic characteristics of this organism include a “horse stable” odor caused by p-cresol production and yellow fluorescence with Wood’s lamp illumination [14]. *C. difficile* form hearty spores that are resistant to heat and desiccation;
tative virulence factor) and mutations in these isolates are extremely rare [24, 25]. Some strains do not produce either toxin and are thus nonpathogenic.

**PATHOGENESIS**

The *C. difficile* genome has been fully sequenced, but not all of the molecular mechanisms by which *C. difficile* causes CDI are completely understood [15]. For example, the mechanics of intestinal colonization and regulatory elements that are important for virulence gene expression are poorly described, compared with those of other gastrointestinal pathogens. However, the toxins responsible for mediating the bulk of the clinical manifestations of infection have been identified and extensively characterized. *C. difficile* toxin A (TcdA) and *C. difficile* toxin B (TcdB) are encoded by the *tcdA* and *tcdB* genes, respectively [16–19]. These proteins share 63% amino acid homology and are glucosyltransferases that inactivate GTP-binding proteins (RHO subfamily) involved in cell cytoskeleton organization and other cellular functions [20, 21]. This activity is the primary mechanism by which these toxins act to alter intestinal permeability and mediate the characteristic inflammation associated with CDI (pseudomembranous colitis) [21].

Both TcdA and TcdB are capable of intoxicating intestinal epithelial cells in vitro and causing intestinal pathology when administered to animals. Using infection models, studies designed to investigate the relative contribution of these toxins to pathogenesis have been contradictory; one study showed that TcdB but not TcdA was required for virulence, whereas another study demonstrated that both toxins are individually capable of causing fatal infection in the *C. difficile* hamster model [22, 23]. Most clinical isolates of *C. difficile* are capable of expressing both TcdA and TcdB, whereas a subset produce only TcdB; TcdA+ TcdB- clinical isolates are extremely rare [24, 25]. Some *C. difficile* strains do not produce either toxin and are thus nonpathogenic. These may asymptomatically colonize the GI tract and must be distinguished from toxigenic isolates by diagnostic assays.

In 2005, a prominent epidemic strain of *C. difficile* (BI/NAP1/O27) was identified. Characteristic genotypic findings in these isolates include the presence of 2 additional toxin genes (*cdtA* and *cdtB*) that encode the *C. difficile* binary toxin (CDT, a putative virulence factor) and mutations in *tcdC* that may dysregulate the expression of *tcdA* and *tcdB* [26]. Phenotypically, these isolates are resistant to fluoroquinolones. Although genetic subtyping of *C. difficile* isolates is possible using a variety of methods, and the performance of some test methods may be influenced by genotype, there is currently no evidence that genotypic characterization of *C. difficile* is useful for clinical management of CDI [27].

**CLINICAL PRESENTATION AND DIAGNOSTIC METHODS**

The clinical features of CDI are often difficult to distinguish from those of other causes of antibiotic-associated diarrhea. Most patients present with diarrhea that has a characteristic “horse stable” odor. Although odor recognition may heighten the suspicion for CDI and prompt early isolation, it is not in itself diagnostically accurate enough to guide therapy [7]. In some cases, radiologic imaging or the presence of a brisk leukocytosis in the absence of evidence of infection at other sites may be helpful in distinguishing CDI from other causes of diarrhea [7]. Prompt CDI identification is required for proper and rapid treatment to prevent disease progression and for timely infection control interventions to reduce the incidence of additional nosocomial cases. According to recent Infectious Diseases Society of America and Society for Healthcare Epidemiology of America guidelines, CDI diagnostic criteria are as follows: diarrhea (defined as passage of ≥3 unformed stools in ≤24 consecutive hours) and a stool test result positive for the presence of toxigenic *C. difficile* or its toxins; or colonoscopic or histopathologic findings demonstrating pseudomembranous colitis [28].

Microbiologic or endoscopic (with or without histologic examination of biopsied tissue) examinations may be used to meet the suggested diagnostic criteria for CDI. Although data defining the performance characteristics of endoscopy are limited, it is estimated that 90% of patients with macroscopic or microscopic evidence of pseudomembranous colitis also have microbiologic evidence of CDI [29–31]. Because endoscopy presents inherent risk to patients, is costly, and is not uniformly available, it should be used sparingly for CDI diagnosis. The American College of Gastroenterology recommends endoscopy for CDI diagnosis when a rapid diagnosis is required and laboratory testing may be delayed, a stool sample is not available from a patient with ileus, or other colonic diseases that can be diagnosed by endoscopy are being considered [32].

There are several diagnostic modalities currently employed in the microbiology laboratory for CDI detection. Some of them target the organism itself, such as culture or the glutamate dehydrogenase (GDH) antigen assay; others detect the presence of *C. difficile* toxins in the stool, such as the cytotoxicity neutralization assay (CCNA) and enzyme immunoassays (EIAs); whereas others detect the presence of the toxin genes (molecular methods). Because *C. difficile* is a normal component of the bowel flora in neonates and asymptomatically colonizes adults, microbiologic testing is only recommended for patients >1 year of age with symptoms that are consistent with CDI and who have a recent history of antibiotic use. A detailed description of...
these methods follows, and a summary of the relative attributes of each of these methods is presented in Table 1.

**Toxigenic Culture**

Although impractical for clinical use, selective media is available for *C. difficile* stool culture [14]. Improvements in culture methods to increase recovery of *C. difficile* from stool specimens have included heat or alcohol shock pretreatment techniques and liquid broth enrichment steps [33]. Because of the imperfect selectivity of available media and the fact that both toxigenic and nontoxigenic (ie, nonpathogenic) *C. difficile* may be isolated from stool, all suspected isolates must be subcultured, and a confirmatory test must be performed to detect the presence of toxin genes or expression of toxin proteins. Obviously, this approach is very time consuming and lengthy, with turn-around times approaching 1 week. Thus, stool culture for *C. difficile* is largely employed in a research setting, even though it does demonstrate some performance benefits over many more-rapid assays [34, 35].

**CCNA**

A direct cell cytotoxicity assay that relies on neutralization of the *C. difficile* toxin B using an antitoxin to enhance specificity was developed soon after the discovery of *C. difficile* in 1978 [36]. This method detects toxin at picogram levels in stool specimens and is performed by adding a prepared stool sample (diluted, buffered, and filtered) to a monolayer of cultured cells. If *C. difficile* toxin B is present, it has a cytopathic effect characterized by rounding of cells in tissue culture. This test is considered to have positive results if characteristic changes are seen in R50% of cells at 48 h and the effect is inhibited by *C. difficile* antitoxin. Interpretation of the results of this assay is necessarily subjective and requires a skilled reader. CCNA has historically been used as the gold standard with which other assays are compared, especially toxin EIAs. However, its sensitivity may be as low as 67% when compared with results obtained by stool culture techniques [35]. Another major limitation of CCNA is that the turn-around time, especially for negative results, is unacceptably long (up to 72 h from sample receipt in the laboratory).
EIAs

EIAs designed to detect C. difficile TcDA were first developed >25 years ago [37]. Since then, assays which detect TcDA, TcDB, the bacterial antigen GDH, or a combination of some or all of these targets have become the most widely used diagnostic approach for CDI in US clinical microbiology laboratories [38]. Compared with CCNA or toxigenic culture, these assays offer rapid turn-around time, are less laborious, and can be performed by technologists that lack advanced training in cell culture techniques. Unfortunately, it has become clear more recently that these assays, especially those designed to detect toxins, lack adequate sensitivity for sole use as a diagnostic modality [39–43]. EIAs that detect GDH exhibit better sensitivity but are less specific, because GDH is expressed by both toxigenic and nontoxigenic C. difficile isolates, as well as by closely related Clostridium species. Assays that detect combinations of these targets (GDH plus TcDA and/or TcDB) generally demonstrate better overall performance [44].

Molecular Methods

Nucleic acid amplification tests (NAATs) for C. difficile were first developed >15 years ago, but interest in their clinical use has recently increased as the clinical and epidemiologic importance of CDI has become more widely appreciated [45, 46]. NAATs employing both real-time polymerase chain reaction (PCR) and loop mediated isothermal amplification of DNA technologies for the detection of C. difficile in stool specimens have been approved by the US Food and Drug Administration (FDA) and are being adopted by clinical laboratories. Currently available FDA-approved kits include the GeneOhm (BD), proGastro (Prodesse), GeneXpert (Cepheid), and Illumigene (Meridian) C. difficile assays. In addition, various laboratory-developed tests are also in use in some institutions, most of which are based on PCR methods and designed to detect the C. difficile tcdB gene. These assays generally take several hours to perform.

Numerous studies have been published, most in the past several years, detailing the results of controlled evaluations of the performance of NAATs, compared with that of conventional methods for the diagnosis of CDI [35, 40–42, 44, 47–50]. Overall, it appears that, at least for all of the FDA-approved tests, these assays are in general much more sensitive than are toxin EIAs (~90% vs 40%–80%, compared with molecular or culture methods) and demonstrate high specificity. Additionally, a recent study demonstrated equivalent sensitivity of NAATs and GDH antigen detection, whereas another study revealed that a NAAT was superior to GDH antigen detection [44, 51]. The variability between studies comparing antigen-based detection to NAATs may be a result of differences in genotype prevalence. Tenover et al [27] have shown that the performance of antigen-based methods fluctuates in a genotype-dependent way, whereas the NAAT included in their study did not exhibit the same variability. It is not yet clear whether all molecular approaches are equal, but at least 1 study documented equivalent performance for 2 of the commercially available assays and a laboratory-developed method [52].

Impressive performance characteristics are achieved with a rapid turn-around time using NAATs for C. difficile detection, leading some authors to conclude that molecular approaches represent the obvious best option for C. difficile testing [53]. However, molecular testing for C. difficile is not a complete panacea. These methods detect the genes associated with toxigenic C. difficile in the stool, whereas traditional CCNAs and EIAs detect the presence of C. difficile toxin(s) in the stool. Considering that up to 50% of institutionalized individuals may be asymptomatically colonized by toxigenic C. difficile and that diarrhea may have a variety of causes, false-positive results are likely, especially given the high sensitivity of molecular approaches [2]. Because it is likely that asymptomatic colonization by toxigenic C. difficile protects patients from CDI, inappropriate therapy under these circumstances may put the patient at greater risk for CDI at a later time. In addition, a significant proportion of patients who have been successfully treated for CDI may have persistent asymptomatic C. difficile colonization for many weeks. Thus, the detection of C. difficile in the stool using NAATs following therapy for laboratory-confirmed infection is not useful. It is clear that, at the least, clinicians must be very careful in selecting appropriate patients to test when using molecular assays.

A final drawback of molecular testing for CDI is cost. FDA-approved NAAT assays may cost 10 times more than an EIA or CCNA (or both together), whereas laboratory-developed methods are more affordable. Laboratories must often make difficult choices to balance test performance and cost; without a large body of data demonstrating cost savings associated with molecular testing for CDI, it will be hard to justify implementation of such an assay in many centers. Limited data do exist showing that the rapid identification of infected patients using an accurate C. difficile assay with a rapid turn-around time may lead to cost savings by impacting patient management and improving infection control practices [35, 44].

Combination Methods and Algorithms

The combination of multiple testing technologies using assays with complementary strengths and weaknesses has been effectively employed in a number infectious disease diagnostic approaches. A variety of algorithms employing EIAs, CCNAs, and/or molecular methods to detect toxigenic C. difficile have been implemented in some laboratories (Figure 1). Many of these algorithms employ a first step that relies on the rapid, sensitive,
inexpensive GDH assay followed by a second step that ensures specificity (a second EIA, CCNA, or molecular method) \[38, 51, 54\]. The performance of such approaches has been extensively evaluated and, depending on the specific assays employed, achieves sensitivities of 75% –100%, compared with molecular methods or toxigenic culture, and has excellent specificity \[35, 38, 40, 42, 44, 51, 53, 55–57\]. The turn-around time for most negative results (ie, GDH negative specimens, which represent 75% - –80% of specimens in most studies) using such algorithms are very rapid (minutes to hours after receipt, depending on laboratory work flow), whereas positive results and GDH-positive or second-test negative results may take no additional time (with concurrent toxin EIA as the second test) or up to 48 h (CCNA as the second test).

Another approach to \textit{C. difficile} testing that may be used in some laboratories is algorithms that employ multiple tests performed in parallel rather than sequentially. For example, EIAs can be performed concurrent with a NAAT. The performance of these approaches has yet to be extensively evaluated.

\textbf{Repeat Testing}

Multiple studies have demonstrated that repeat stool testing is ineffective for the diagnosis of CDI \[58–60\]. For example, Aichinger et al \[60\] demonstrated that repeat testing for CDI within a 7-day period following a negative test result obtained using either an immunoassay or a NAAT resulted in just 1.9% and 1.7% diagnostic gains, respectively. Because no \textit{C. difficile} assay or algorithm is 100% specific, a small percentage of false-positive results should be expected. When repeat testing is performed for CDI within a 7-day period, the pre-test probability for the second assay is so low that the ratio of true-positive results to false-positive results becomes very unfavorable; this could result in misdiagnosis for some patients.

\textbf{IMPLICATIONS FOR CLINICAL PRACTICE}

In a study conducted at our institution (University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School; New Brunswick, NJ) in 2000, El-Gammal et al \[61\] demonstrated that testing for CDI did not significantly impact treatment decisions, and empirical treatment for CDI was continued whether laboratory results were positive or negative. This observation appears to reflect that relatively little weight is given to laboratory test results for \textit{C. difficile} when integrating them with the clinical picture. This could result from uncertainty on the part of the clinician regarding the performance characteristics of the assay, delays in availability of results, and/or poor communication of results.

Given the variability in performance characteristics and the limitations and strengths of the available tests for \textit{C. difficile}, it is obvious that test methods must be known when interpreting results. It is important to consider that the practical value (negative and positive predictive values) of tests performed at a given institution not only depends on the tests employed but also on analytical (positivity cut-off values, for example, in laboratory-developed or modified FDA-approved assays and the technical skill of those who perform the tests) and pre-analytical variables (such as specimen handling protocols and the population-specific prevalence of disease). Clinical microbiology consultation is warranted if a physician is unsure of current laboratory practice. In addition, because the significance of

\textbf{Figure 1.} Typical diagnostic algorithm for the detection of toxigenic \textit{Clostridium difficile} in stool specimens. Rapid, inexpensive immunoassays that detect glutamate dehydrogenase (GDH) (and may also include toxin detection as well; Step 1) are followed by more-laborious and/or more-expensive approaches that often demonstrate better performance characteristics (Step 2). Specimens with negative results in the first step (which often represent >80% of specimens) and those that are GDH positive as well as \textit{C. difficile} toxin A (TcdA) and/or \textit{C. difficile} toxin B (TcdB) positive may be reported after the first step, allowing most laboratories to achieve a very favorable turn-around time for the bulk of specimens. Those that are positive for GDH (and negative for toxin(s) if tested) are further assessed using a nucleic acid amplification test (NAAT) or cell cytotoxicity neutralization assay (CCNA). Although NAATs are often more expensive than CCNAs, the performance and turn-around time for NAATs are superior to those for CCNAs.
positive test results will depend on whether a patient is likely to be asymptomatically colonized, as opposed to actively infected, with *C. difficile*, a proper selection of which patients to test and careful consideration of the overall clinical picture is extremely important. Specifically, only those patients meeting clinical criteria for CDI should be tested, and thus only unformed stool specimens should be accepted for *C. difficile* testing by the laboratory. Armed with a knowledge of test characteristics and an awareness of the current practice of the laboratory to which they submit specimens, physicians can more confidently integrate test results with clinical data to formulate a rational therapeutically approach.

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**References**


