Egyptian Journal of Medical Microbiology Volume 25 / No.3 / July 2016 53-58

REVIEW ARTICLE

Laboratory Diagnosis of *C.difficile* Infection

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INTRODUCTION

The clinical features of *C. difficile infection* (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. 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[1].

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 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[2].

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 Gastroeneterology 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[3].

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 cell cytotoxicity neutralization assay (CCNA) and enzyme immunoassays (EIAs) whereas others detect the presence of the toxin genes. 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[4].

Specimen collection:

The proper laboratory specimen for the diagnosis of *C. difficile* infection is a watery, loose, or unformed stool promptly submitted to the laboratory. Except in rare instances in which a patient has ileus without diarrhea, swab specimens are unacceptable, because toxin testing cannot be done reliably. Because 10% or more of hospitalized patients may be colonized with *C. difficile,* evaluating a formed stool for the presence of the organism or its toxins can decrease the specificity of the diagnosis of CDI. Processing a single specimen from a patient at the onset of a symptomatic episode usually is sufficient. Because of the minimal increase in yield and the possibility of false-positive results, routine testing of multiple stool specimens is not supported as a cost-effective diagnostic practice[5].

Methods used for diagnosis of CDI:

I. Culture:

The name “*C.difficile”* reflects the difficulty encountered by investigators to isolate and grow these *Clostridia* on conventional media. Culture has been a mainstay in the laboratory diagnosis of CDI and is essential for the epidemiologic study of isolates. The description of a selective agar medium that includes egg yolk which may be replaced by 5% sheep blood and contains cycloserine, cefoxitin, and fructose (CCFA medium) provided laboratories with a selective culture system for recovery of *C. difficile*[6].

Addition of taurocholate or lysozyme can enhance recovery of *C. difficile,* presumably because of increased germination of spores. Optimal results require that culture plates be reduced in an anaerobic environment prior to use. 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. After anaerobic incubation for 48-72h at 33-37C° the strains produce flat, grey white to yellow, ground glass–appearing colonies with a surrounding yellow halo in the medium (Figure 1). The colonies

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have a typical odour and fluoresce with a Wood's lamp. Additionally, Gram staining of these colonies show typical morphology (gram-positive or gram-variable bacilli) for *C. difficile*[4].

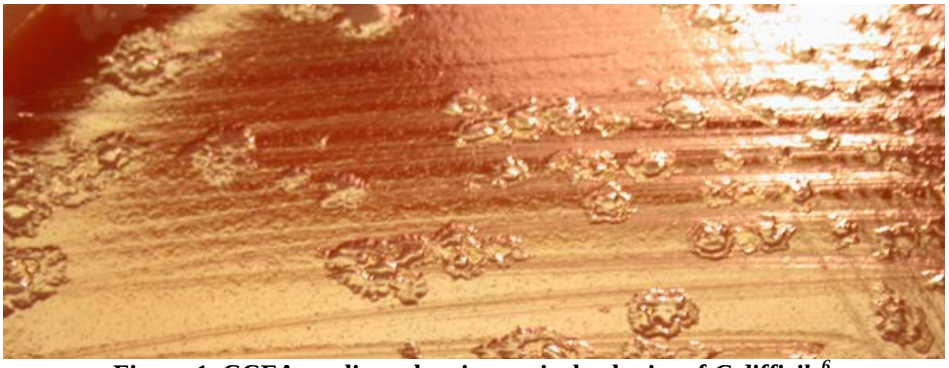

Figure 1. CCFA medium showing typical colonies of *C.difficile*[6].

Careful laboratory quality control of selective media for isolation of *C. difficile* is required, as there have been variations in the rates of recovery with media prepared by different manufacturers. With experience, visual inspection of bacterial colonies that demonstrate typical morphology on agar and confirmation by Gram stain usually is sufficient for a presumptive identification of *C. difficile*. Isolates not fitting these criteria can be further identified biochemically or by gas chromatography[2].

Both toxigenic and nontoxigenic (i.e., non-pathogenic) *C.difficile* may be isolated from stool that is why a confirmatory test must be performed to detect the presence of toxin genes or toxin proteins. This process is known as "toxigenic culture". Colonies of *C.difficile* obtained are further subcultured anaerobically on Wilkins Chalgren agar supplemented with egg yolk or 5% sheep blood for 24h at 33-37C□, followed by toxin protein detection by EIA or toxin gene detection by PCR. Obviously, this approach is very time consuming and lenghthy, with turn- around time approaching one week. Thus, stool culture for *C.difficile* is largely employed in a research setting, even though it does demonstrate performance benefits over many more-rapid assays[4].

II. Cell Cytotoxicity Neutralization Assay:

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*. 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 ≥ 50% 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. Cell cytotoxicity neutralization assay (CCNA) has historically been used as the gold standard with which other assays were compared, especially toxin EIAs. However, its sensitivity may be as low as 67% when compared with results obtained by toxigenic stool culture techniques. Another major limitation of CCNA is that the turnaround time, especially for negative results, is unacceptably long (up to 72 h from sample receipt in the laboratory)[7].

III. Enzyme Immuno- Assay (EIA):

a. EIA for Toxins A and B:

Commercial EIA tests have been introduced that either detect toxin A only or detect both toxins A and B. The toxin A/B assay is preferred because 1%–2% of strains in the United States are negative for toxin A. Compared with diagnostic criteria that included a clinical definition of diarrhea and laboratory testing that included CCNA and toxigenic culture the sensitivity of these tests is 63%–94%, with a specificity of 75%–100%. In addition, these assays offer rapid turn-around time, are less laborious, and can be performed by technologists that lack advanced training in cell culture techniques. These tests have been adopted by more than 90% of laboratories in the United States[2].

b. EIA for C. difficile Common Antigen Glutamate Dehydrogenase (GDH):

EIAs that detect GDH exhibit better sensitivity but are less specific, because GDH is also expressed by closely related Clostridium species. In addition, GDH is present in both toxigenic and nontoxigenic *C. difficile* isolates. Assays that detect combinations of these targets (GDH plus TcdA and/or TcdB) generally demonstrate better overall performance[8].

The initial test developed to detect GDH was a latex agglutinin assay. It had a sensitivity of only 58%–68% and a specificity of 94%–98%. Even though it is rapid, relatively inexpensive, and specific the latex test for *C. difficile*–associated antigen is not sufficiently

sensitive for the routine laboratory diagnosis of CDI. In addition, the use of this test provides no information regarding the toxigenicity of the isolate, nor does it yield the isolate itself, which would be useful for epidemiologic investigations[2].

Several assays for GDH have been developed using EIA methodology. These newer assays show a sensitivity of 85%– 95% and a specificity of 89%–99%. Most importantly, these tests have a high negative predictive value, making them useful for rapid screening, if combined with another method that detects toxin. Several 2-step algorithms have been developed that are based on the use of this test. They all use the GDH test for screening in which a stool sample with a negative assay result is considered negative for the pathogen but a positive assay result requires further testing to determine whether the *C. difficile* strain is toxigenic[6].

One of the studies performed 2-step testing of 5,887 specimens at 2 different hospitals. The GDH test result was positive for 16.2% of specimens at one hospital and 24.7% of specimens at the other. Therefore, 75%–85% of the samples did not require that a cell cytotoxicity assay be performed, at a cost savings of between $5,700 and $18,100 per month.

Swindells *et al.* stated that EIA-based assays for *C.difficile* toxins and GDH lack adequate sensitivity for sole use as a diagnostic modality[10].

IV. Molecular Methods:

Nucleic acid amplification tests (NAATs) for *C. difficile* were first developed > 15 years ago, but interest in their clinical use has increased as the clinical and epidemiologic importance of CDI has become more widely appreciated. NAATs employing both real-time 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 2008, the FDA approved the first commercially available real-time PCR assay (the BD GeneOhm C.diff assay; BD Diagnostics, San Diego, CA) to directly detect the toxin B gene in stool to aid in the diagnosis of CDI. R-Biopharm (Darmstadt, Germany) offers a group of Conformite Europeene – in vitro diagnostic (CE-IVD) marked RIDA GENE-Gastrointestinal kits which utilise multiplex real-time PCR for diagnosis of *C.difficile* gene and toxins genes. 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[4, 11].

Numerous studies have been published, most in the past several years, evaluating the performance of NAATs, compared with that of conventional methods for the diagnosis of CDI[7, 10, 12]. 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%). Sharp *et al.* demonstrated equivalent sensitivity of NAATs and GDH antigen detection[8], whereas Novak-Weekly *et al.* revealed that a NAAT was superior to GDH antigen detection[13]. The variability between studies comparing antigen-based detection to NAATs may be a result of differences in genotype prevalence.

Tenover *et al.* 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[14].

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. However, molecular testing for *C. difficile* is not a complete solution. These methods detect the genes associated with toxigenic *C. difficile* in the stool[15]. 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. 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[16].

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[7, 8].

A summary of the relative attributes of the different detection methods is presented in (Table 1).

Table 1: Advantages and Disadvantages of *Clostridium difficile* Assays[4].

Assay	*Method/target*	*Advantages*	*Disadvantages*
Culture	Organism	High sensitivity (often considered to be the gold standard)	Turn-around time >7 days Labor intensive Lacks specificity; does not distinguish between toxigenic and nontoxigenic strains Isolates must be further tested for the presence of toxin(s) or toxin genes (toxigenic culture)
Cell cytotoxicity neutralization assay	Functional assay for *C. difficile* toxin B (TcdB)	Moderate-to-high sensitivity High specificity	48-72 h turn-around time Subjective interpretation; requires skilled technicians/ technical expertise Labor intensive
Enzyme immunoassays (EIA), C. difficile toxin A (TcdA)	Toxin A detection	Easy to perform Rapid turn-around time Inexpensive High specificity	Low sensitivity Misses TcdA-/TcdB+ isolates
EIA, TcdA/B	Toxin A/B detection	Easy to perform Rapid turn-around time Inexpensive High specificity	Low sensitivity
EIA, glutamate dehydrogenase	Common antigen detection	High sensitivity Good screening test	Low specificity and does not distinguish between toxigenic and nontoxigenic strains Positive specimens must be further tested for the presence of toxin(s) or toxin genes
Nucleic acid amplification tests	Toxin gene(s) detection	High sensitivity and specificity (new gold standard?) Short turn-around time Some easy to perform and minimal hands-on time	Expensive when used to test all samples Detection of asymptomatic colonization is a possible concern Some require significant molecular expertise

V. Combining Methods and Algorithms:

The combination of multiple testing technologies using assays with complementary strengths and weaknesses has been effectively employed in a number of 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. 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)[17,13].

One example of the algorithms implemented is illustrated in figure 2.

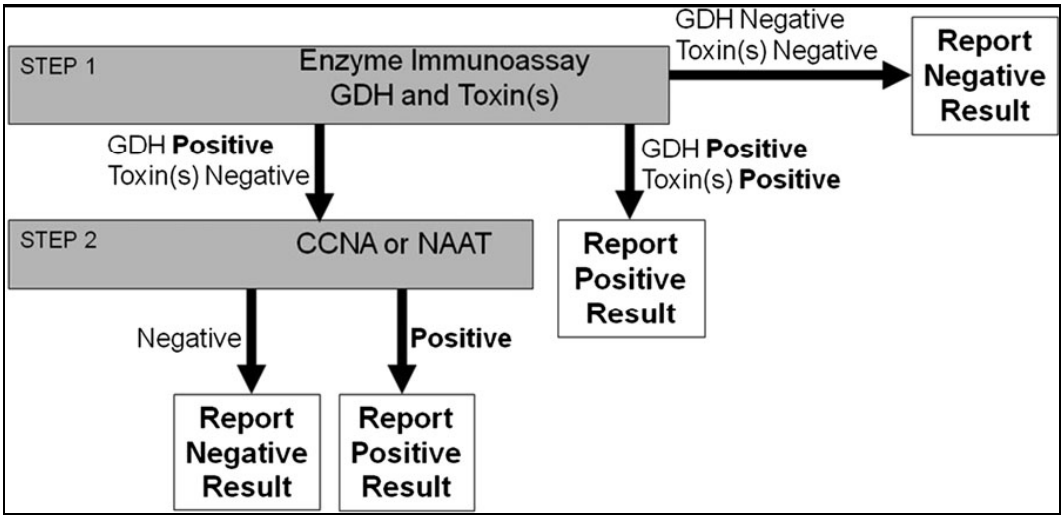

Figure 2. Typical diagnostic algorithm for the detection of toxigenic *Clostridium difficile* in stool specimens[4].

Rapid, inexpensive immunoassays that GDH and toxins (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 *C. difficile* toxin A (TcdA) and/or *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.

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. 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) [13,8,15].

Repeat Testing:

Multiple studies have demonstrated that repeat stool testing is ineffective for the diagnosis of CDI. **Aichinger *et al.*[5]** 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 *C. difficile* assay or algorithm is 100% specific, a small percentage of false-positive results should be expected.

REFERENCES

1. Bartlett, J. G. and Gerding, D.N. (2008): Clinical Recognition and Diagnosis of *Clostridium difficile* Infection. *Clin. Infec. Dis.*46 (1): 12-18
2. Cohen, S.T., Gerding, D.N., Johnson, S. *et al.* (2010): Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect. Control. Hosp. Epidemiol*. 31(5)
3. Thielman, N.M. and Wilson, K.H. (2010): Antibiotic-Associated Colitis. *In*: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. Mandell G.L., Bennett J.E., Dolin R. (eds): 7th ed. Vol 1. Philadelphia, PA: Churchill Livingstone/Elsevier, 2010
4. Kufelnicka, A.M. and Kirn, T.J. (2011): Effective Utilization of Evolving Methods for the Laboratory Diagnosis of *Clostridium difficile* Infection. *Clin.Infect.Dis.*52:1451-1457
5. Aichinger, E., Schleck, C.D., Harmsen, W.S., *et al.* (2008): Nonutility of Repeat Laboratory Testing for Detection of *Clostridium difficile* by Use of PCR or Enzyme Immunoassay. *J. Clin. Microbiol.* 46:3795–3797
6. Reller, M.E., Lema, C.A., Perl ,T.M., *et al.* (2007): Yield of Stool Culture with Isolate Toxin Testing Versus a Two-Step Algorithm Including Stool Toxin Testing for Detection of Toxigenic *Clostridium difficile*. *J. Clin. Microbiol.*45:3601–3605

7. Stamper, P.D., Alcabasa, R., Aird, D., *et al.* (2009): Comparison of a Commercial Real-Time PCR Assay for tcdB Detection to a Cell Culture Cytotoxicity Assay and Toxigenic Culture for Direct Detection of Toxin-Producing *Clostridium difficile* in Clinical Samples. *J. Clin. Microbiol.* 47:373–378
8. Sharp, S.E., Ruden, L.O., Pohl, J.C. *et al.* (2010): Evaluation of the C.Diff Quik Chek Complete Assay, A New Glutamate Dehydrogenase and A/B Toxin Combination Lateral Flow Assay for Use in Rapid, Simple Diagnosis of *Clostridium difficile* Disease. *J. Clin. Microbiol.* 48:2082–2086
9. Ticehurst, J.R., Aird, D.Z., Dam, L.M., *et al.* (2006): Effective Detection of Toxigenic *Clostridium difficile* by a Two-Step Algorithm Including Tests for Antigen and Cytotoxin. *J. Clin. Microbiol.* 44:1145–1149.
10. Swindells, J., Brenwald, N., Reading, N., *et al.*, (2010): Evaluation of Diagnostic Tests for *Clostridium difficile* Infection. *J. Clin. Microbiol.* 2
11. Reddington, K., Tuite, N., Minogue ,E., *et al.* (2014): A current overview of commercially available nucleic acid diagnostics approaches to detect and identify human gastroenteritis pathogens. *Biomol. Detect. Quant* .1: 3–7. 48:606–608.
12. Larson, A.M., Fung, A.M. and Fang, F.C. (2010): Evaluation of tcdB Real-Time PCR in a Three-Step Diagnostic Algorithm for Detection of Toxigenic *Clostridium difficile*. *J. Clin. Microbiol.* 48:124–130.
13. Novak-Weekley, S.M., Marlowe, E.M., Miller, J.M., *et al.* (2010): *Clostridium difficile* testing in the clinical laboratory by use of multiple testing algorithms. *J. Clin. Microbiol*. 48:889–893.
14. Tenover, F.C., Novak-Weekley, S., Woods, C.W., *et al.(*2010): Impact of Strain Type on Detection of Toxigenic *Clostridium difficile*: Comparison of Molecular Diagnostic and Enzyme Immunoassay Approaches. *J. Clin. Microbiol*. 48:3719–3724.
15. Wilcox, M.H., Planche, T. and Fang, F.C. (2010): What is The Current Role of Algorithmic Approaches for Diagnosis of *Clostridium difficile* Infection? *J. Clin. Microbiol*.48:4347–4353.
16. Riggs, M.M., Sethi, A.K., Zabarsky, T.F., *et al.* (2007): Asymptomatic Carriers are a Potential Source for Transmission of Epidemic and Nonepidemic *Clostridium difficile* Strains among Long-Term Care Facility Residents. *Clin. Infect. Dis.* 45:992–998
17. Schmidt, M.L., Gilligan, P.H. (2009): *Clostridium difficile* Testing Algorithms: What is Practical and Feasible? *Anaerobe.* 15:270–273.