

**Clostridium difficile. A review on an emerging infection**

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**Abstract**

*Clostridium difficile* causes antibiotic-associated diarrhoea and pseudomembranous colitis. The main virulence factors of *C. difficile* are the toxins A (TcdA) and B (TcdB). A third toxin, binary toxin (CDT), which pathogenetic role had been remained largely overlooked until few years ago, nowadays have been detected in 5%-23% of strains. *C. difficile* has spread around world. *Clostridium difficile* infection (CDI) is one of the most common health-care associated infections and a significant cause of morbidity and mortality among older adult hospitalized patients. Diagnosis of CDI is often difficult and has a substantial impact on the management of patients with disease. It is usually based on a clinical history of recent antimicrobial usage and diarrhoea in combination with laboratory tests. Although the conventional methods are crucial for the diagnosis and the subsequent treatment of CDI, a timely laboratory diagnosis is essential for the detection of toxigenic strains providing either to an effective and immediately treatment or to the prevention of further disease transmission.

In this review we provide general recommendations for testing of samples collected from patients with suspected CDI. *Clin Ter* 2019; 170(1):e41-47. doi: 10.7417/CT.2019.2106

**Key words:** *Clostridium difficile* infection, diagnostic methods, therapy

**Background**

This work is a review of the strategies that may be used for laboratory diagnosis of *Clostridium difficile* infection (CDI). Worldwide, CDI represents an important public health problem. European and American studies report an increasing of CDI in terms of incidence, prevalence, morbidity and mortality (1). CDI causes a wide spectrum of disease, ranging from mild to severe diarrhoea (2-3) due to cytotoxic power of their enzymes (*C. difficile* toxin A: TcdA and *C. difficile* toxin B: TcdB). Both the toxins are potent monoglucosyltransferases, active on small GTP-binding proteins (Rho, Rac and Cdc42) involved either in the regulation or in the formation of the cytoskeletal actin in the intestinal epithelium (4-5). In addition, some *C. difficile* strains produce a third toxin (Binary toxin, CDT), composed of an enzymatic component (CDTa) and a binding component (CDTb). CDTb binds to a cell receptor leading to the internalization of CDTa into the cytosol catalysing the ADP-ribosylation of monomeric actin and the resultant disruption of the actin cytoskeletal (6-7). The majority of *C. difficile* strains harbouring the binary toxin genes are also A+ B+ (A+ B+ CDT+). Several studies have reported that the production of CDT in addition to TcdA and TcdB by *C. difficile* is associated with severe disease, higher mortality and an elevated risk of recurrence in humans, suggesting that CDT might play an important role in disease pathogenesis (8). Among A+ B+ CDT+ strains, the most prevalent type is the epidemic PCR ribotype 027/toxinotype III, also known as 027/B1/NAP1 (9). This ribotype has an 18-base pair deletion at nt117 of TcdC, a negative regulator of expression either of TcdA or TcdB, therefore there is an iper-production of TcdA and TcdB (10). In the last decade, this strain has increased in Europe, USA, Canada and Asia (11). In Italy, it actually represents around the 9% of the toxigenic strains isolated in hospitalized patients (12).

Recently, some studies have highlighted an increase of *C. difficile* strains producing the binary toxin, despite the lack of cdtA gene (A- B+ CDT+) (13-14). This strain is the epidemic PCR ribotype 036/toxinotype X (15). Interestingly, Geric et al. and Eckert et al. have described also some strains (about 2%) producing the binary toxin but negative for cdtA and cdtB genes (A- B- CDT+) (16-17). Among A- B- CDT+ strains, the most prevalent type is the epidemic PCR ribotype 033/toxinotype Xa/b (18-19). In the last ten years, the incidence of diseases associated with the new emerging ribotypes has increased either in Europe or in other Countries (USA, Canada and Asia), therefore, the timely laboratory diagnosis is crucial for the treatment of CDI (11, 20-21).

The diagnosis of CDI should be based on a combination of clinical and laboratory findings.
CDI should be suspected in patients with acute diarrhoea (>3 loose stools in 24 hours), particularly in the setting of relevant risk factors (including recent antibiotic use, hospitalization and advanced age) (22). Although the conventional methods are crucial for the diagnosis and the subsequent treatment of CDI caused by TcdA and TcdB producing strains, a timely laboratory diagnosis is essential for the detection of hypervirulent strains providing either to an effective treatment immediately or to the prevention of further disease transmission (1).

The aim of this review is to briefly provide general recommendations for testing of samples taking in account the most accepted guidelines for diagnosis, treatment and prevention of CDI (23).

**Conventional methods**

Previously, the stool cytotoxicity (CTA) and the toxigenic culture (TC) methods had been used in the diagnosis of CDI (24-26). The CTA is sensitive and specific but it is relatively slow, time-consuming and expensive for the necessity of maintaining cell lines. For regards the TC, it is either very slow (48 to 72 h) or laborious. Moreover, the test is not able to identify the non-toxigenic strains. Therefore both conventional methods are unlikely to be adopted by a clinical laboratory as the standard methods for C. difficile testing. As of today, most laboratories have adopted enzyme immunoassays (EIAs) for toxins A and B as the routine method of testing (27).

**Multistep algorithm for the laboratory diagnosis of CDI**

According to the The European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the diagnosis of CDI recommends the use of a two or three-step algorithm. The first step consists of either a glutamate dehydrogenase enzyme immunoassay (GDH-EIA) or the nucleic acid amplification test (NAATs) as screening test. Samples resulting from the first step can be reported as negative CDI, whereas those with positive results should be confirmed (toxins-EIA). Subsequently, the samples confirmed by this second step can be reported as positive CDI (28).

A number of laboratory stool tests are available alone or in combination as part of a diagnostic algorithm:
- GDH-EIA
- toxins EIA
- NAATs

**GDH-EIA**

The C. difficile produces and secretes GDH (highly conserved enzyme), which allows to the bacterium to limit the oxidative stress derived from inactivating hydrogen peroxide through the production of ketoglutarate (29). For this assay, several studies have shown a sensitivity of 85-95% and a specificity of 89-99%, underlining in particular a high negative predictive value, making it useful for a rapid screening (30-31) (Tab.1). However, its value is limited because it is not able to discriminate between toxigenic and non-toxigenic strains (32).

**Toxin- EIA**

Although some strains produce only toxin B, most C. difficile strains produce both toxins A and B (33-37). No CDI due to strains producing toxin A alone has been reported.

In the last two decades, the toxins EIA has been among the most widely used for diagnosing CDI for their rapid and inexpensive performance despite their poor sensitivity (38). In fact, this assay shows a sensitivity of around 60% and a better specificity (up to 99%), though several studies report the presence of false positives values associated to assay (39-41) (Tab.1). Moreover, it is important to note that C. difficile toxin degrades at room temperature and might be undetectable within two hours after collection; therefore, specimens should be kept at 4°C. To overcome these limits, the NAATs represent the best method to detect the toxigenic strains.

**NAATs**

NAATs detect one or more specific genes of toxigenic strains; the critical gene is tcdB, which encodes for toxin B. NAATs are highly sensitive (42-44) compared to EIA (38, 45-46).

NAATs are specific for toxigenic strains but do not test for active toxin protein production and are capable of detecting asymptomatic carriers of C. difficile leading to either an overdiagnosis of CDI or an antibiotic treatment of patients who may not require such therapy with subsequently overestimation of hospital CDI rates (47). In fact, Polage et al. in their study on more than 1400 patients with suspected CDI have demonstrated that patients whose stool were positive by NAAT but negative by immunoassay had a lower toxin load and less diarrhoea than patients for whom both assays were positive (48). For circumstances in which initial testing consisted of NAAT (with positive result) is appropriate subsequent testing with the toxins EIA to bolster the clinical specificity.

Currently, real-time PCR (RT-PCR) assays have been commercially developed in order to overcome the lack of sensitivity of EIA. The RT-PCR designed to detect the conservative region of tcdB within the locus of pathogenicity (PaLoc) are: Quidel Lyra Direct C. difficile™ assay [Quidel], ProdGastro™ Cd [Prodesse], BD GeneOhm Cdiff™ [Becton

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**Table 1. Results in terms of sensitivity and specificity from different assays.**

<table>
<thead>
<tr>
<th>Assays</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH-EIA</td>
<td>85-95%</td>
<td>89-99%</td>
<td>Carman RJ et al., 2012</td>
</tr>
<tr>
<td>Toxin EIA</td>
<td>60,00%</td>
<td>99,00%</td>
<td>Alcala L et al., 2008</td>
</tr>
<tr>
<td>Illumigene™</td>
<td>77-97%</td>
<td>93-100%</td>
<td>Coyle K et al., 2010</td>
</tr>
<tr>
<td>Cepheid Xpert™</td>
<td>90-100%</td>
<td>92.9-98.6%</td>
<td>Huang H et al., 2009</td>
</tr>
</tbody>
</table>
Dickinson], and Xpert™ C. difficile [Cepheid]. They were cleared by the Food and Drug Administration (FDA) for U.S. laboratory use. In Europe, the Xpert C. difficile assay targets tcdB in combination with binary toxin and deletion of tcdC for the presumptive identification of the 027 clone. This assay does not have FDA clearance for distribution in the United State, whereas it is commercially available in Europe. Several clinical studies have shown that these assays exhibit the best concordance with EIA and therefore could represent a promising alternative for the diagnosis of CDI (38, 49-54) (Tab.1).

Another amplification assay (illuminigene™ C. difficile, Meridian Bioscience, Cincinnati, OH) is based on the original loop-mediated isothermal amplification (LAMP) technology. This assay detects the PaLoc by targeting a DNA fragment in the 5' region of the tcdA, which is intact in all strains, including those with a large deletion in the tcdA gene. Coyle et al. recently showed that Illumigene C. difficile was positive in stools spiked with A’ B’ strains (55). Many clinical trials have recently evaluated the performance of the real-time PCR-based methods currently available on the market. Their sensitivity and specificity range from 77.3% to 97.1% and 93% to 100%, respectively (38, 49-54, 56). The performance characteristics of these Illumigene C. difficile assays are in agreement with those data, with sensitivity and specificity of 91.8% and 99.1%, respectively (Tab.1). Moreover the technology is isothermal contrary to the RT-PCR, therefore requires no costly capital equipment.

More recently, there are a variety of multiplex syndromic platforms (Luminex xTAG GPP, Luminex Molecular Diagnostics and BioFire FilmArray GI Panel, BioFire Diagnostics) able to detect the main causative agents of diarrhoea among bacteria, viruses and protozoa. These assays have been performed on a multiplex reverse transcriptase PCR technology (57-58).

**Treatment**

The choice of antibiotic therapy should be tailored to the severity of disease presentation. The common antibiotics used for non severe disease are either oral metronidazole or vancomycin. Among their, metronidazole is usually recommended for treatment of mild-moderate disease, whereas oral vancomycin is generally preferred (24, 59-60). The vancomycin is poorly absorbed from the gastrointestinal tract, therefore luminal drug levels are high leading to either a more rapid suppression of C. difficile or a faster resolution of diarrhoea, contrary to metronidazole (61-62). However, both oral metronidazole and oral vancomycin have associated with the persistent overgrowth of vancomycin-resistant enterococci (VRE) in stool samples obtained from colonized patients during CDI treatment, increasing the risk of transmission (63). Moreover, they cause a significant destruction of the commensal microbiota, predisposing to intestinal colonization of VRE and Candida spp.

An alternative treatment of mild/moderate disease is the use of Fidaxomicin, which appears to cause either less disruption of the microbiota or a lower risk of colonization by VRE (64). For regards the severe disease, a recent study have suggested an anti-IP-10 antibody, BMS-936557, as a potentially effective therapy (65).

According to ESCMID guidelines, the use of high doses of vancomycin (500 mg four times daily) is recommended for the management of severe complicated CDI, while the patients with fulminant CDI require surgical intervention (colectomy). In fact, several studies suggest that earlier colectomy is associated with improved survival (66). Recently, an alternative surgical approach involves the laparoscopic creation of a loop ileostomy and colonic lavage. This procedure involves creating a loop ileostomy, with intra-operative colonic infusion and lavage with warmed poly-ethylene glycol solution and post-operative instillation of vancomycin flushes antegradely (67).

Recurrent CDI presents when the return of symptoms occurs within 8 weeks of the previous episode. About 10-20% of CDI recur after an initial episode of C. difficile, but when a patient has had one recurrence, rates of further recurrences increase to 40-65% (68). For the treatment of the first CDI recurrence, ESCMID recommends the same therapeutic drug used in the initial episode, while for the multiple recurrent CDI unresponsive to repeated antibiotic treatment, faecal transplantation in combination with oral antibiotic treatment is strongly recommended (69).

**Emerging therapy for C. difficile hypervirulent strains:** Studies performed since 2000 in Europe, Canada and the United States have shown an association with fluoroquinolones exposure and infection with hypervirulent strains (70-74). On basis of the antibiotic associations, these studies have somewhat different prevalences about strains, but a meta-analysis supports the fluoroquinolones use as a risk factor for infection with PCR ribotype 027. This is occurring because the fluoroquinolones are a class of antibiotics with a wide spectrum of activity used extensively and inappropriately to treat a great variety of infections (75-76). These findings have obvious implications for antibiotic stewardship interventions, but further studies in settings where this strain predominates are needed.

Nowadays, CRS3123 is becoming a promising therapy for the treatment of emerging ribotypes (77). CRS3123 is a therapeutic agent that selectively inhibits the growth, spore and toxin production of C. difficile. CRS3123 has not demonstrated any cross resistance to existing antibiotics remaining active against all C. difficile strains, above all against the epidemic fluoroquinolones-resistant NAP1/BI/027 strains.

**Conclusion**

C. difficile has a worldwide distribution and its toxigenic strains are responsible of clinically relevant infections. In the last decade, despite the knowledge on risk factors responsible of CDI, in many countries there was however an increase in terms of prevalence (78-82).

Nowadays, CDI is one of the most common healthcare-associated infections and it is responsible of morbidity and mortality increased among adult hospitalized patients. Among risks factors, the most important is represented by inappropriate antibiotics usage, especially broad-spectrum antibiotics, that destroy the intestinal microbiota exacerbating the disease.
One of the challenging in the management of CDI is the early diagnosis. To date, the most widely used tests in clinical microbiology laboratories for detection of CDI have not adequate sensitivity as stand-alone tests (83). For this reason, a multistep testing which includes NAATs is recommended for a better diagnosis combining high sensitivity with a short turnaround time (3). Moreover, a timely and accurate laboratory diagnosis for CDI is important also to limit the nosocomial spread of *C. difficile* in healthcare settings. In fact, the main objective of laboratory tests is to identify cases of CDI excluding those not due to CDI, since diarrhoea is the most frequent symptom, caused by infectious and non-infectious agents, in hospitalized and long-term care patients. Therefore, a proper diagnosis of CDI reduces transmission and prevents either inadequate or unnecessary treatments.

Some limitations in this review must be accounted. Firstly, it is a narrative review, thus an evaluation of the methodological quality of the cited studies by a standardized scoring tool has not performed and also the articles selection could be potentially biased. Secondly, among all the most frequently diagnostic methods reported in literature, just a few have been described. An accurate selection of the diagnostic tests must be tailored to each laboratory needs in order to optimize sensitivity, specificity and turnaround time.

Finally, it is important to highlight that the provided laboratory results must be interpreted with caution because they must be associated with clinical data and accurate evaluation of risk factors of CDI in order to exclude the presence of a non pathological *C. difficile* colonization. Further studies will be needed to improve the knowledge of the pathogenesis of CDI providing to the clinicians new strategies to overcome the actual limits of the laboratory diagnosis.

**Conflict of interest:** The authors declare that they have no conflict of interest to disclose.

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