World Journal of Gastroenterology

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World J Gastroenterol 2017 March 7; 23(9): 1552-1567

DOI: 10.3748/wjg.v23.i9.1552

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Current knowledge on the laboratory diagnosis of *Clostridium difficile* infection

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Manuscript source: Invited manuscript

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Received: November 15, 2016
Peer-review started: November 15, 2016
First decision: January 10, 2017
Revised: January 21, 2017
Accepted: February 16, 2017
Article in press: February 17, 2017
Published online: March 7, 2017

Abstract

Clostridium difficile (*C. difficile*) is a spore-forming, toxin-producing, gram-positive anaerobic bacterium that is the principal etiologic agent of antibiotic-associated diarrhea. Infection with *C. difficile* (CDI) is characterized by diarrhea in clinical syndromes that vary from self-limited to mild or severe. Since its initial recognition as the causative agent of pseudomembranous colitis, *C. difficile* has spread around the world. CDI is one of the most common healthcare-associated infections and a significant cause of morbidity and mortality among older adult hospitalized patients. Due to extensive antibiotic usage, the number of CDIs has increased. Diagnosis of CDI is often difficult and has a substantial impact on the management of patients with the disease, mainly with regards to antibiotic management. The diagnosis of CDI is primarily based on the clinical signs and symptoms and is only confirmed by laboratory testing. Despite the high burden of CDI and the increasing interest in the disease, episodes of CDI are often misdiagnosed. The reasons for misdiagnosis are the lack of clinical suspicion or the use of inappropriate tests. The proper diagnosis of CDI reduces transmission, prevents inadequate or unnecessary treatments, and assures best antibiotic treatment. We review the options for the

laboratory diagnosis of CDI within the settings of the most accepted guidelines for CDI diagnosis, treatment, and prevention of CDI.

Key words: *Clostridium difficile*; Toxigenic culture; Nucleic acid amplification tests; Enzyme immunoassay; Diagnosis; Glutamate dehydrogenase

Core tip: This work is a review of the strategies that may be used for laboratory diagnosis of infection with *Clostridium difficile*. First, we provide general recommendations for testing of samples taking in account the guidelines of the Society for Healthcare Epidemiology of America/Infectious Diseases Society of America and the American College of Gastroenterology. We reviewed diverse methods of diagnosis including, culture, toxigenic culture, cell cytotoxic neutralization assay and the use of enzyme immuno assays. Finally, we present an overview of singleplex and multiplex nucleic acid amplification tests.

Martínez-Meléndez A, Camacho-Ortiz A, Morfin-Otero R, Maldonado-Garza HJ, Villarreal-Treviño L, Garza-González E. Current knowledge on the laboratory diagnosis of *Clostridium difficile* infection. *World J Gastroenterol* 2017; 23(9): 1552-1567 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1552.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1552

INTRODUCTION

Clostridium difficile (*C. difficile*) is a Gram-positive and strictly anaerobic bacterium that may exist in a vegetative form that is very sensitive to oxygen. During stress, *C. difficile* produces spores that enable the microbe to survive harsh conditions for prolonged periods of time and facilitate its dissemination in the environment[1]. Upon ingestion, the spores resist the low pH of the stomach and reach the anaerobic environment of the gut. When the intestinal microbiote is altered because of antibiotic treatment, especially broad-spectrum antibiotics, the spores germinate. Next, *C. difficile* develops into its vegetative form, proliferates, and colonizes the gut[2]. *C. difficile* infection (CDI) is the principal cause of antibiotic-associated diarrhea. Diarrhea because of CDI may be self-limited, mild, or severe, and is one of the symptoms of a variety of clinical syndromes due to CDI. Complications of CDI are pseudomembranous colitis, fulminant colitis, and toxic megacolon[3].

CDI is an intestinal disease mediated by potent cytotoxic enzymes that damage the intestinal mucosa[4,5]. These cytotoxic enzymes, toxin A (TcdA) and toxin B (TcdB)[6,7], alter cytoskeletal actin, which leads to diminished transepithelial resistance, fluid accumulation, and destruction of the intestinal epithelium[5,8]. *C. difficile* toxins also induce the release of pro-inflammatory cytokines from enterocytes, mast cells, and macrophages[9]. The genome of toxigenic strains of *C. difficile* has a pathogenicity locus (PaLoc) of 19.6 kb that contains the *tcdA* and *tcdB* genes. Other PaLoc genes are *tcdR* and *tcdC*; the former encodes a positive regulator and the latter a negative regulator of the expression of the A and B toxins. Yet another PaLoc gene is *tcdE*, which encodes a holin-like protein that may be involved in the secretion of toxins[10]. Besides, some strains produce the *C. difficile* binary toxin (CDT), which is composed of an enzymatic component, CdtA, and a binding component, CdtB. These components are codified by *cdtA* and *cdtB* genes, which are located in the CDT locus (CdtLoc). CDT may potentiate the toxicity of TcdA and TcdB and lead to a more serious illness and could, therefore, be considered another virulence factor[11,12].

The vast majority of diarrhea cases are not related to a particular pathogen. From all the stool samples submitted to the laboratory for testing of *C. difficile* toxins, only 10% to 25% are positive[13]; most commonly in cases of antibiotic-associated diarrhea. Other pathogens that may cause antibiotic-associated diarrhea are: *Staphylococcus aureus*, *Clostridium perfringens*, *Salmonella* species, and *Klebsiella oxytoca*[14]. The clinical suspicion of CDI is the presentation of diarrhea after administration of antibiotics shortly after the beginning of treatment and up to 8 wk after treatment initiation[13]. In mild to moderate disease, diarrhea is the main symptom and passing of watery stools with foul odor is characteristic. The presence of hematochezia is rare. Moderate to severe disease is usually accompanied by systemic symptoms such as abdominal cramps, fever (up to 40 ℃), leukocytosis (up to 50000 cells/mm^3), and hypoalbuminemia (< 2.5 mg/dL)[13,15]. Colitis is characterized by fever, cramps, leukocytosis, and the presence of leukocytes in feces. Furthermore, a thickened colon wall is observed with computed tomography and in half of the cases pseudomembranes can be seen with endoscopy[13]. When pseudomembranes are found, a diagnostic of CDI can be made, as antibiotic-associated diarrhea due to other pathogens tends to have normal endoscopy findings[16]. In severe cases, CDI may progress to toxic megacolon with the risk of colon wall perforation[15]. The diagnosis of toxic megacolon is accomplished through radiological evidence of colonic dilatation, commonly involving the ascending or transverse colon[17]. According to the most commonly used criteria for the diagnosis of toxic megacolon[18], three of the following four criteria should be present: fever, tachycardia, leukocytosis, and anemia. Additionally, dehydration, electrolyte disturbance, and hypotension or changes in mental status (any of the criteria must be present)[17-19].

Ever since *C. difficile* was recognized as the caus-

ative agent of pseudomembranous colitis, the number of CDI cases has increased worldwide. Recently, Lessa *et al*[20] estimated that the number of CDI in the United States was 453000, from which 29000 died within 30 d after diagnosis. The healthcare costs related to CDI were estimated to be $ 4.8 billion for acute care facilities alone[20]. Since the year 2000, both the number and severity of CDI have increased due to the emergence of a more virulent strain with a higher antimicrobial resistance[21]. After strain typing by pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis, and ribotyping, this strain was denominated BI/North American PFGE type 1 (NAP1)/027[21]. So far, all BI/NAP1/027 isolates are positive for binary toxin CDT and have an 18-base pair deletion in *tcdC* that is associated with an increased production of toxins A and B; a single base pair deletion at position 117 of *tcdC* has also been related to higher toxin expression[3]. Concerning antimicrobial resistance, BI/NAP1/027 are resistant to fluoroquinolones, which provides a selective advantage to this strain[21]. This strain is disseminated worldwide; the above is evidenced by reports from America, Europe, Asia, and Oceania[22].

One of the key points when treating CDI is to discontinue unnecessary therapy with antibiotics; thus allowing the gut microbiota to recover. The Clinical Practice Guidelines for *C. difficile* Infection in Adults of the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), the Guidelines for Diagnosis, Treatment, and Prevention of *C. difficile* Infections of the American College of Gastroenterology (ACG) and the Guidance Document for *Clostridium difficile* of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommend the use of metronidazole when treating an initial episode of mild to moderate CDI. The dosage is 500 mg orally, 3 times per day for 10 to 14 d. In case of severe initial CDI, vancomycin (125 mg orally, 4 times per day for 10 to 14 d) should be administered[23-25]. A combination of oral vancomycin (500 mg 4 times per day) and intravenous metronidazole (500 mg every 8 h) are indicated for the treatment of severe, complicated CDI according to the SHEA/IDSA and ESCMID guidelines[23,25]. On the other hand, the ACG recommends a dosage of 125 mg of vancomycin. When treating a first episode of recurrence, the regimen should be the same as an initial case, according to the severity of the infection. If there is a second episode of recurrence, a pulsed regimen of vancomycin is recommended. Fidaxomicin is narrow spectrum macrocyclic antibiotic, approved by the American Food and Drug Administration (FDA), that selectively eradicates *C. difficile* with a minimum effect on the intestinal microbiota. The relapse rate of fidaxomicin is lower than the one of vancomycin[26-28].

Despite the high burden of CDI and the increased interest in the disease, episodes of CDI are often misdiagnosed. A study from Spain revealed that two out of three CDI cases were either undiagnosed or misdiagnosed[29]. The main explanation may be the lack of clinical suspicion, particularly in community cases with patients who do not meet the risk criteria (age > 65 years or previous hospitalization). Besides, an inadequate test may yield false-negative results[30]. Furthermore, the interpretation of laboratory data is complicated, as the presence of *C. difficile* in stool does not mean CDI; the other way round, the absence of *C. difficile* toxins does not rule out the possibility of CDI. To interpret laboratory results, the techniques that were applied must be considered. A correct diagnosis of CDI is important because it has a substantial impact on case managment, mainly with regards to antibiotic regimens. The diagnosis of CDI is primarily based on the clinical signs and symptoms and is only confirmed by laboratory testing[31]. Misdiagnosis has two main consequences: first, patients may be undertreated or overtreated; second, the delay of proper infection control allows for further dissemination[32].

The following is a review of the strategies that may be used for laboratory diagnosis of CDI. To date, the Clinical Practice Guidelines of the SHEA/IDSA provide one of the most widely acceptable guidelines for the diagnosis and clinical management of CDI cases[23]. Also, the Guidelines of the ACG focus on the recommendations for the diagnosis and management of patients with CDI as well as for the prevention and control of outbreaks[24]. The above documents provide useful recommendations on which this review is based.

GENERAL RECOMMENDATIONS

Recommended samples

Both the Clinical Practice Guidelines of the SHEA/IDSA and the Guidelines of the ACG state that *C. difficile* testing is recommended only for stool samples from patients with diarrhea (Table 1), which is defined as the evacuation of loose stools, three or more times in 24 h or less[23,24]. The ESCMID recommends testing only stools of Bristol score 5 to 7[25]. The Bristol scale is a graded visual scale, composed of seven grades which range from stools with a form of separate hard lumps (score 1) to watery stools (score 7)[33]. To correlate the Bristol scale with *C. difficile* detection, stool samples with Bristol scale $\geqslant$ 5 were tested for *C. difficile* with an enzyme immunoassay (EIA) for glutamate dehydrogenase (GDH) and toxins A/B followed by a molecular assay for indeterminate results[34]. Detection of *C. difficile* was more frequent in semiformed stools (Bristol 5 or 6) than in watery stools (Bristol 7). Bristol 5 stool specimens accounted for the highest rate of positive testing. Therefore, Bristol 5 stool specimens should not be discarded. Further study is required to verify whether specimen with lower Bristol scores should be tested. There was no association between the Bristol score and the rates of hospital-onset CDI, severe CDI, and complications of CDI[34].

Commonly, perirectal swabs are not accepted for

Table 1 General recommendations for *Clostridium difficile* testing

Recommendation	Ref.
C. difficile testing is recommended only for stool samples from patients with diarrhea	[23,24]
The testing of asymptomatic patients is not recommended	[23]
Perirectal swabs are not accepted for *C. difficile* testing unless the patient has developed ileus	[23,24]
Repeated testing of *C. difficile* does not improve detection and does not change the result	[23,24]
Retesting, as a proof of cure, remains controversial	[23,24]

C. difficile: *Clostridium difficile*.

C. difficile testing, except for selected cases, such as patients with ileus[23,24]. Ileus is characterized by a lack of bowel movements that causes a blockage of the intestines. For patients with ileus, an accurate sampling technique is the perirectal swab[35]. Some patients with CDI develop fever and abdominal pain but not diarrhea. These patients may develop severe complications, like fulminant colitis and intestinal perforations[36].

Patients to evaluate

The Clinical Practice Guidelines of the SHEA/IDSA recommend no testing of asymptomatic patients[23] (Table 1) because asymptomatic colonization with a toxigenic strain of *C. difficile* is common. But even samples from patients with persistent diarrhea may yield false-positive CDI results; for example, alternate etiology was reported to be the cause of the symptoms in 25% of a cohort of 117 cases that had been diagnosed with recurrent CDI[37]. Particularly, highly sensitive molecular assays may yield false-positive results. Positive CDI tests for asymptomatic patients are common. The carriage rates of toxigenic *C. difficile* strains are similar between open populations (6.6%)[38] and hospitalized ones (8%)[39]. Hospitalized populations tend to be at an increased risk of developing CDI due to antibiotic treatment and prolonged exposure because of long stays at healthcare facilities[40]. The environment and skin of asymptomatic carriers have higher percentages of spores and represent a permanent source of contamination and spreading of spores to other patients and setting surfaces. The continual washing of hands of both personnel and patients is a universal preventing measure[41].

The carriage rate of *C. difficile* is high among infants (0 to 3 years of age). Among 85 healthy infants at day nurseries, the carriage of *C. difficile* was 45%, and the frequency of toxigenic strains was 13%[42]. One-year follow-up studies among newborns revealed that 74% to 100% had CDI-positive stool, often in the neonatal period[42] In a study that followed 10 infants, 81/111 samples (73%) were positive for *C. difficile* and 21 (26%) had toxigenic strains[42]. Another study that followed 42 infants found that 106/288 stool samples (37%) were CDI positive[43]. Most strains (71%) were toxin producers. Interestingly, six infants evacuated loose stools during the study, but only three of them could be linked to *C. difficile*. Furthermore, carriage of *C. difficile* was similar in infants suffering from loose stools than children with normal stools.

A point to consider when testing specimens from infants is the difficulty to differentiate a diarrheal stool from a normal stool, since infant stool may not be fully formed. When comparing infant cases (age $\leqslant$ 12 mo) with CDI-positive diarrheal stool with cases with CDI-negative diarrheal stool no differences in clinical symptoms were found. However, in both groups, alternative causes of diarrhea were found[44]. Rather than looking for *C. difficile*, the authors suggested looking for other causes, especially viral ones, of diarrhea in infants.

Retesting of samples

Neither the Clinical Practice Guidelines of the SHEA/IDSA nor the Guidelines of the ACG recommend retesting (Table 1). This recommendation is especially valid for molecular methods. Several studies have demonstrated that repeated testing for *C. difficile* does not improve detection nor change the result. Repeated testing, particularly with molecular tests, only increases healthcare costs and the probability of false-positive results. After having implemented a new policy that alerted physicians about the consequences and disadvantages of a *C. difficile* PCR test within 7 d of an initial test, a healthcare institute saw the requests for CDI retesting reduced by 91%[45]. Among the 135 retests that were performed, 122 were repeaters after a negative initial test, and only 4 of them turned positive upon repeating the test. Even lower positive conversion rates (0.05%-1%) have been reported by others after repeating PCR assays on 4213 samples with negative results in a previous test[46]. Thus, an initial test with a negative test result does not justify retesting unless there is a founded suspicion of a false-negative result.

Retesting to monitor response to treatment also remains controversial. Although a negative conversion rate of 67.6% 14 d after a positive Cepheid Xpert *C. difficile* test has been reported[47], cases that were clinically cured remained positive when testing for toxins[48].

DIAGNOSTIC TESTS

Culture

A stool culture is essential to prepare isolates for molecular typing, which is required for epidemiologic studies. The SHEA/IDSA guidelines recommend toxigenic culture (TC) as the standard to which other methods should be compared. The first step is to recover *C. difficile* spores. Stool samples are either heated to 80 ℃ or mixed with an equal volume of

absolute ethanol and incubated at room temperature. This way vegetative cells and contaminating microbes are eliminated while spores are recovered[49]. Next, the sample is inoculated into a differential and selective medium that allows the spores to germinate. A well-known medium to recover *C. difficile* from stool specimens is cycloserine-cefoxitin-fructose-agar (CCFA)[50]. Cycloserine and cefoxitin are present at concentrations that inhibit the growth of most Gram-negative and Gram-positive bacteria, without affecting the growth of *C. difficile*. Fructose is an important nutrient and neutral red is added as a pH indicator. A 48-h solid culture of *C. difficile* in CCFA presents flat, grayish, and shiny colonies with spreading edges and a typical horse manure smell. Under ultraviolet light, the colonies appear yellow-green fluorescent. Under the microscope, cells of *C. difficile* are Gram-positive and possess subterminal to terminal spores. Identification of *C. difficile* colonies may be based on colony morphology, Gram staining, and odor; confirmation of species may be assessed trough biochemical systems for the identification of anaerobes.

To improve both the recovery and identification of *C. difficile* cultures, the original CCFA formulation has been modified. The addition of biliary salts, particularly sodium taurocholate, promotes germination[51] and the inclusion of egg yolk allows to verify lecithinase and lipase activity of isolates[50,52]. Furthermore, enrichment broths have been formulated to recover small amounts of spores and allow them to germinate. When comparing two broths, a cycloserine-cefoxitin fructose broth proved to be more sensitive than a cycloserine-cefoxitin mannitol broth that had been supplemented with taurocholate and lysozyme, (CCMB-TAL), but CCMB-TAL yielded better recovery rates when cultures were semi-quantified[49].

Furthermore, chromogenic media have been developed. For example, *C. difficile* grown on ChromID *C. difficile* agar (bioMérieux, France) yields black colonies that often can be observed after 24 h of incubation[53]. However, lengthening the incubation from 24 to 48 h increased the sensitivity significantly from 53% to 100% ($P < 0.001$)[54]. ChromID *C. difficile* agar yields a higher 24-h recovery (sensitivity, 92%) than CCFA (sensitivity, 22%)[55]. Likewise, ChromID *C. difficile* agar, which had a sensitivity of 100% and a recovery of 94%, outperformed CCFA supplemented with sodium taurocholate, which had a sensitivity of 87% and a recovery of 82%[56]. Yet another study confirmed that ChromID *C. difficile* agar performs best when compared to CCFA, cycloserine-cefoxitin-egg-yolk agar and tryptone soy agar with sheep blood[49].

Toxigenic culture

TC is a two-step reference method for the diagnosis of CDI. In step one, *C. difficile* strains are isolated and grown on a selective medium, and in step two, colonies are tested for toxin production on a variety of cell lines. The grown isolates are re-cultured in broth, and the supernatant is filtered and added to a cell line culture. The cytopathic effect (CPE) is evaluated and neutralized by antitoxin. This procedure may take a few days to accomplish, which makes it an impractical option for routine diagnosis[57]. Alternatively, testing of toxin production may be performed using an EIA[58,59].

The main concern about TC is the possibility of recovering non-toxigenic strains (strains that are not capable of produce toxins A and/or B). Another possibility is, though recovering a toxigenic strain, it may not be producing toxins, thus not causing clinical symptoms. When evaluating the clinical significance of TC and the citotoxicity assay on 169 samples that met CDI criteria, it was found that cases positive for both assays were more severe than cases that were positive for TC only. On the other hand, if only the cytotoxicity assay had been performed, one-third of the cases would have been missed[60]. The latter is an argument in favor of TC for CDI diagnosis.

Cell Cytotoxic Neutralization Assay

The Cell Cytotoxic Neutralization Assay (CCNA) has been considered the gold standard for the diagnosis of CDI. In this assay, the filtrate of a recently obtained stool sample is inoculated onto various sensitive cell lines to evaluate the CPE of *C. difficile* toxins, particularly TcdB[57]. CPE is observed as cell rounding; some strains may induce protrusions in the cell lines, a phenomenon known as "sordellii-like" CPE[61,62]. If the CPE can be reversed by an antitoxin, the test is positive for the *C. difficile* toxin[57]. The assay must be performed in fresh stools, since sample freezing or a delay in its processing may result in loss of activity of toxins and false negative test results[63].

Diverse cell lines have been used for the detection of toxins: African green monkey kidney, McCoy, MRC-5, primary rhesus monkey kidney, and Vero cells. Among these cell lines, Vero cells and McCoy cells were a 100% concordant with respect to the detection of toxins[64]. When maintaining a cell line is impossible or non-desirable, there are cost-effective commercial assays available. A CCNA executed with Hs27 HFF ReadyCells is not only easy-to-use but also outperforms the EIA and TC in both sensitivity and specificity; 90.8% *vs* 78.6% in sensitivity and 98.3% *vs* 97.8% in specificity, respectively[65].

Glutamate dehydrogenase assay

C. difficile produces and secretes GDH; this enzyme allows the bacterium to manage oxidative stress derived from the immune response by inactivating hydrogen peroxide through the production of α-ketoglutarate[66]. Although GDH screening of stool specimens for the diagnosis of CDI diagnosis is common[67], its value is limited to being a preliminary test, since both toxigenic and non-toxigenic strains produce GDH[68]. GDH is highly conserved among *C. difficile*

strains; no differences in reactivity were found among 168 *C. difficile* isolates belonging to 77 different ribotypes using three different assays carried out by two different groups[68]. As the GDH assay may be positive for *C. difficile* strains that do not produce toxins, a positive GDH assay needs a confirmatory test (CCNA, EIA, a molecular test, or TC)[23,35,67].

GDH has been incorporated, as an initial test, into multistep algorithms (ACG guidelines). Recently, there has been a tendency towards a two-step algorithm, where in the absence of detection of toxin by EIA, clinical evaluation should also be applied to determine true CDI or colonization. This has recently been recommended by ESCMID[69].

The C. Diff Quik Chek Complete assay is a rapid membrane EIA that combines the detection of both GDH and the toxins A and B[70]. With TC as a reference, the C. Diff Quik Chek Complete had a sensitivity of 63.6% and a specificity of 98%[70]. Both assays proved to be highly sensitive (range: 97.6% to 100%) and accurate (88% true positive or true negative). Discrepant results were resolved with the Xpert C. diff assay[71]. The algorithm allowed to rule out *C. difficile* without additional tests when GDH is negative and to confirm CDI when both GDH and toxin A/B results are positive. Another GDH test, the automated Vidas *C. difficile* GDH assay (bioMérieux, Marcy l'Etoile, France), has a 95% agreement with the C. Diff Quik Chek assay (Quik Chek-60, Techlab, United States)[72]. In case of discrepant results, molecular tests are recommended by various authors, because CCNA and TC are expensive and time-consuming[35,73]. A two-step algorithm (step 1, GDH and toxin detection by EIA; step 2, loop-mediated isothermal amplification) yielded a sensitivity of 81%, a specificity and positive predictive value (PPV) of 100%, and a negative predictive value (NPV) of 96%[74]. Even though the sensitivity was lower than in other studies, the PPVs and NPVs supported the practice of reporting the specimen as positive without further testing. A more complex algorithm increased the CDI detection rate from 8% to 19%; initial positive GDH testing (VIDAS *C. difficile*; bioMérieux) was confirmed by toxin testing (VIDAS *C. difficile* Toxin A&B; bioMérieux). In case a toxin EIA did not confirm a positive GDH, additional tests [nucleic acid amplification test (NAAT), CCNA, or TC] were executed on positive GDH samples. A positive confirmatory test is considered CDI-positive sample[75].

Apart from providing improved diagnostic performance, multistep algorithms are the most cost effective for various reasons: (1) because they avoid unnecessary or inadequate treatment and its consequences[76]; and (2) an initial screening with a cheap GDH test allows rapid identification of negative samples, limiting the use of more expensive NAAT tests to only those samples that were positive for GDH[77].

Detection of toxins by enzyme immunoassays

One of the first strategies for the diagnosis of CDI was the detection of toxins with a specific EIA. Several EIA-based kits are commercially available in different formats, such as: lateral flow immunoassay, also known as immunochromatography or strip tests; and solid-phase assays, for example micro-wells[15]. EIA-based confirmation of CDI is practical, fast, and cheap, but it is also one of the least consistent methods (sensitivity range: 63% to 94%, specificity range: 75% to 100%)[23]. According to the SHEA and IDSA guidelines, toxin detection by EIA is less sensitive than detection by CCNA; thus, EIA should not be used alone for the diagnosis of CDI to avoid a false-negative result[23]. A two-step or three-step algorithm that combines GDH screening with EIA improves CDI diagnosis[23,24].

The inconsistent sensitivity of EIAs may be due to several factors, such as: antigenic variation among the toxins of different circulating strains, inadequate storage and transportation of samples, freeze-thaw cycles, and inter-laboratory technical variance, among others[15]. Some of the early developed EIAs accounted only for the detection of toxin A; however, there are reports of strains that do not produce toxin A[78-80]. Also, some assays detect both toxins A and B plus the detection of GDH[81].

Using TC and CCNA as reference, six commercially available EIAs and three lateral-flow assays for the detection of *C. difficile* toxins A and B have been compared. The sensitivities ranged from 60% to 81.6%, whereas the specificities ranged from 91.4% to 99.4%. PPVs and NPVs were diverse and depended on whether the samples originated from a low-prevalence environment (community) or a high-prevalence environment (hospital setting). Though PPVs were low for both settings, the PPV was higher in the high-prevalence setting, independent from the gold standard chosen as a reference. NPVs were high for both settings (above 95%), regardless of the reference chosen[82]. In a two-step algorithm, the initial screening with GDH detection (C.Diff Chek-60, TechLab/Wampole) yielded a sensitivity of 93.4% and a specificity of 96.6% (reference assay: TC). Next, only positive specimens were confirmed with a rapid toxin A/B assay (Tox A/B Quik Chek, TechLab/Wampole, Blacksburg, VA), which yielded a specificity and a PPV of 97.1% and 96.5%, respectively. Compared to TC, the sensitivity of the EIA-based toxin assay was low (52.9%)[83].

Nucleic acid amplification tests

Nowadays, many infections are diagnosed *via* molecular tests. The new generation of NAATs amplify and detect pathogen-specific DNA or RNA sequences. Advantages of NAATs include high sensitivity, high specificity, and speed. Because no viable cells are needed, sampling, handling, transportation, and storage aspects are simplified. Furthermore, no culture is required. The role of NAAT in the diagnosis process for CDI may be supportive[84], part of a two or three-step algorithm according to ESCMID guidelines (Figure 1)[23], or as a stand-alone test in cases of documented

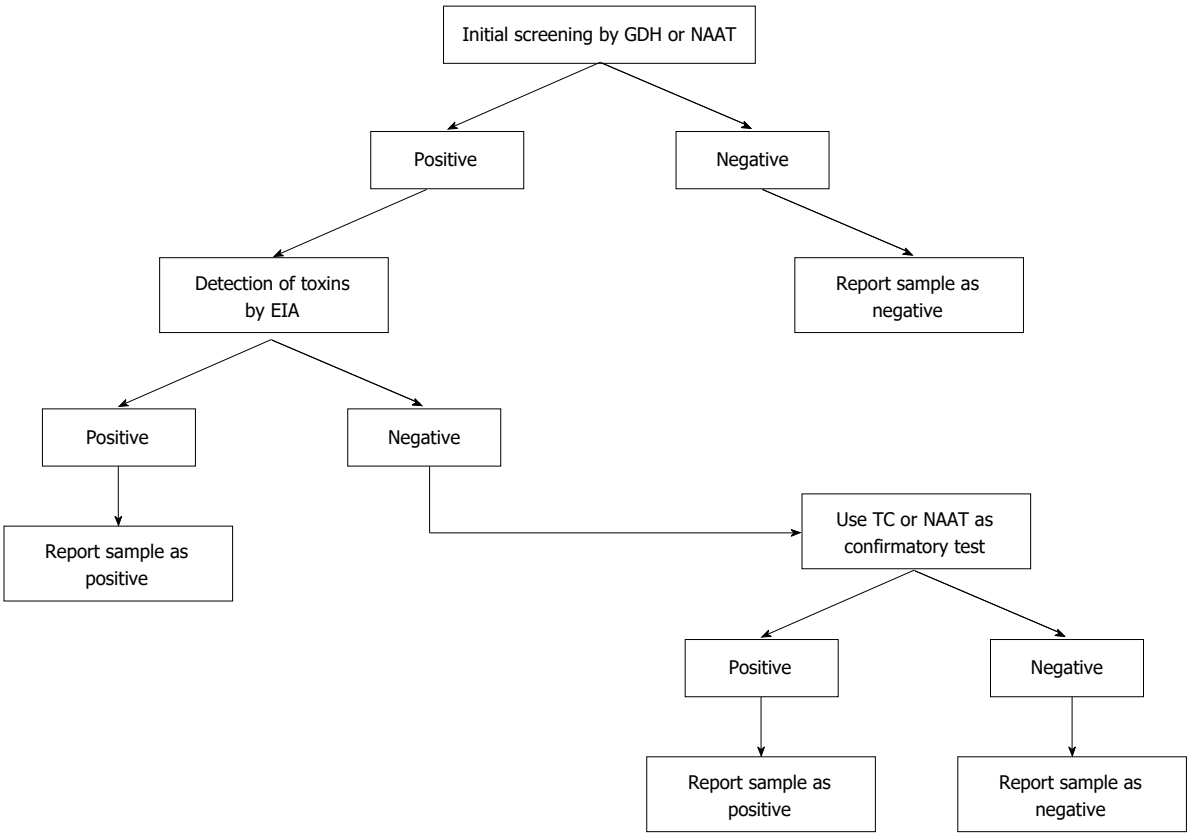

Figure 1 Multistep algorithm for the laboratory diagnosis of *Clostridium difficile* infection based on the European Society of Clinical Microbiology and Infectious Diseases guidance document. GDH: Glutamate dehydrogenase; EIA: Enzyme immunoassay; NAAT: Nucleic acid amplification test; TC: Toxigenic culture.

Table 2 Sensitivity and specificity of nucleic acid amplification test assays for the detection of *Clostridium difficile*

Assay	Sensitivity	Specificity	Ref.
Cepheid Xpert *C. difficile*	90%-100%	92.9%-98.6%	[87,89,91]
IMDx *C. difficile* for Abbott *m*2000 Assay	62.1%-92.8%	99.4%-100%	[92,93]
BD Max Cdiff Assay	81.6%-96.9%	95%-95.8%	[92,93]
Portrait Toxigenic *C. difficile* Assay	98.2%	92.8%	[95]
Quidel Lyra Direct *C. difficile* Assay	82.1%-85.7%	96.9%-98.3%	[96]
Verigene *C. difficile* nucleic acid test	95.2%-98.7%	87.5%-99.4%	[97,128]
Simplexa *C. difficile* Universal Direct real time PCR	87%-98%	100%	[99,128]
AmpliVue *C. difficile* assay	91%-96%	89%-100%	[99,100]
Illumigene *C. difficile* assay	93.3%-100%	95.1%-100%	[95,101]
BD GeneOhm Cdiff assay	89.6%-97.4%	96.7%-98.5%	[95,103]
ProGastro Cd assay	77.93%-100%	93.4%-99.2%	[103,104]

C. difficile: *Clostridium difficile*.

diarrhea[24].

Despite the evident advantages, important issues to be considered before introducing CDI NAATs in the clinical laboratory are: the requirement of trained personnel and higher costs, and the probability of false-positive results because of the high sensitivity of the test and the detection of strains that do not produce toxins. Especially in stool samples from diarrhea cases due to other pathogens, false-positive results may not be recognized as such because of concomitant asymptomatic *C. difficile* carriage. Stool samples that were positive for both NAAT and toxin test had more bacteria and toxins than stool samples that were only NAAT positive[85]. The latter is an argument against the use of NAAT as a stand-alone test.

Single-plex NAATs: The FDA of the United States of America has cleared a set of commercially available single-plex NAATs. Table 2 summarizes the sensitivities and specificities of these tests, which often is over 90% or even close to 100%; these values depend on the test that was used as a reference test, which was

often TC.

Cepheid Xpert® *C. difficile* and Xpert *C. difficile*/Epi: The Cepheid Xpert® *C. difficile* (Sunnyvale, CA) is a real-time PCR assay that detects the *tcdB* gene and thus allows CDI diagnosis, but without strain specification[86]. The multiplex RT-PCR assay Xpert *C. difficile*/Epi not only detects the *tcdB* gene but also the binary toxin genes (*cdtA* and *cdtB*), and a single-nucleotide deletion of the *tcdC*. Therefore, the Xpert *C. difficile*/Epi identifies ribotype 027[87]. Furthermore, Xpert *C. difficile*/Epi detects ribotype 033, a strain of veterinary importance that has been reported in cattle, veal calves, piglets, horses, and soil from various geographical locations worldwide[88]. The binary toxins of ribotype 033 were correctly amplified in all isolates (n = 52) included in a study. However, since ribotype 033 lacks the *tcdA* and *tcdB* genes, the GeneXpert Dx system reports that the sample is negative for CDI, and it is necessary to access the raw data in the instrument to obtain the amplification information[88]. Using TC as a reference, the sensitivity of the Cepheid Xpert *C. difficile*/Epi assay was 90%; its specificity, 92.9%; its PPV 71.4%; and its NPV 97.9%[89].

The introduction of the Cepheid Xpert *C. difficile* assay in a tertiary hospital significantly increased the rate of detection of toxigenic *C. difficile* from 4.7% to 9.9%. The increase was mainly due to cases that yielded indeterminate results with the C. Diff Quik Chek, but were positive with the Xpert *C. difficile* assay[90].

When the performance of the Xpert *C. difficile* Epi assay as a confirmatory test was compared to TC in a two-step algorithm with a GDH assay as a first step, there was a moderate agreement (kappa score 0.48) of 72.6%. The GDH-TC algorithm had a sensitivity of 57% and specificity of 97%, whereas the sensitivity of the GDH-Xpert algorithm was 100% and its specificity 97%. Furthermore, 42 out of 45 stool samples that were ribotype 027 positive were confirmed by PCR-ribotyping and sequencing, indicating a good epidemic value of the assay[91].

IMDx *C. difficile* for Abbott *m*2000 assay: The IMDx *C. difficile* for Abbott *m*2000 assay (IMDx) is a real-time PCR that detects not only *C. difficile tcdA* and *tcdB* genes, but also the rare variant strains rare toxin A^+B^- and toxin B variant (*tcdBv*) gene, which occurs in A^-B^+ strains. Lysis of the sample, target amplification, and detection are performed in the *m*2000 RealTime System (Abbott Laboratories, Abbott Park, IL)[92].

In a prospective analysis of 111 stool specimens and a retrospective analysis of 88 stool samples, in which the IMDx was compared to another FDA-cleared NAAT (the GeneOhm Cdiff Assay), the sensitivity was strain dependent: 100% for NAP1 strains and 90.3% for non-NAP1 strain with a limit of detection of 2250 colony-forming units[92]. However, when IMDx and 2 other molecular assays were compared to TC, IMDx had the lowest sensitivity (62.1%) and the highest specificity (99.4%)[93].

BD Max Cdiff assay: The BD Max Cdiff assay detects and amplifies the *tcdB* gene in a real-time PCR assay performed on the BD Max System (BD Diagnostics, Sparks, MD). After the addition of the sample, this hands-free platform combines DNA extraction and amplification. To extract genetic material a 10-μL loop is immersed in the specimen; next, the loop content is dispersed in BD Max Sample Buffer. The DNA extraction utilizes magnetic beads, which are eluted before a lyophilized amplification mix is added. The results are reported only as positive or negative for *C. difficile*[92].

Compared to the GeneOhm Cdiff Assay, BD Max Cdiff Assay had a sensitivity of 96.9% and a specificity of 95%[92]. In the same study, ribotyping was assessed, but there were no significant differences between the sensitivities and specificities of different ribotypes[94]. When the BD Max Cdiff Assay and 2 other molecular assays were compared to TC, the BD Max Cdiff Assay had a sensitivity of 81.6% and a specificity of 95.8%[93].

Portrait Toxigenic *C. difficile* assay: The Portrait Toxigenic *C. difficile* assay (Great Basin, West Valley City, UT) amplifies a 78-nucleotide fragment of the *tcdB* gene. The assay uses isothermal helicase-dependent amplification, followed by detection with an immobilized capture probe on a sliding array. Each reaction mixture contains three controls: a sample processing control, a hybridization control, and a detection control. Results for the specimen are reported only when the detection criteria for all controls are met[95].

A multicenter evaluation that included 49 stool specimens from 4 clinical sites compared the Portrait Toxigenic *C. difficile* Assay to TC on the same specimens. The sensitivity ranged from 92.9% to 100%, with an overall sensitivity of 98.2%. The specificity ranged from 88.9% to 96.9%, with an overall specificity of 92.8%[95]. When comparing Portrait Toxigenic *C. difficile* Assay results with those from other FDA-cleared tests, the concordance were as follows: 97.5% with Xpert *C. difficile*, 96.4% with GeneOhm Cdiff, and 93.8% with Illumigene *C. difficile*[95].

Quidel Lyra Direct *C. difficile* assay: The Quidel Lyra Direct *C. difficile* assay (Quidel, San Diego, CA) uses qualitative real-time PCR technology. Specimens are tested in a standard TaqMan real-time PCR assay utilizing primers/probes that detect but do not distinguish the *tcdA* and *tcdB* genes. The Lyra assay may be performed on any of three open-platform, real-time thermocyclers: SmartCycler II (Cepheid, Sunnyvale, CA), ABI 7500 Fast DX (Applied Biosystems, Carlsbad, CA), and ABI QuantStudio DX (Applied Biosystems, Carlsbad, CA). The Lyra assay has a running time

of about 3 h[96]. Depending on the platform used, the sensitivity and specificity may differ; the ABI 7500 instrument is the most sensitive and the ABI QuantStudio DX is the most specific. The overall sensitivity is 85.7% and the overall specificity is 98.3% when compared to toxigenic culture[96].

Verigene *C. difficile* nucleic acid test: The Verigene *C. difficile* nucleic acid test is a multiplex qualitative assay that amplifies DNA by PCR in a nanoparticle-based microarray that targets the *tcdA* and *tcdB* genes and differentiates the hypervirulent strain 027/NAP1/BI *via* the binary toxin genes and the base pair deletion at position 117 in the regulator *tcdC* gene[97]. The Verigene system contains two modules: the Verigene Processor SP performs nucleic acid extraction, PCR amplification, and hybridization of amplicons; The Verigene Reader scans the test cartridge, realizes the optical analysis, and generates the results[97].

When compared to TC, the Verigene assay has sensitivity of 98.7% and a specificity of 87.5%[97]. With regard to strain typing, the assay assigns correctly 89.7% of hypervirulent strains compared with ribotyping[97]. When compared to fecal culture as a reference method, the Verigene *C. difficile* nucleic acid test was sensitive (96.7%), specific (97.4%), and accurate (97.1%)[98].

Simplexa *C. difficile* Universal Direct real-time PCR: This assay uses fluorescent bifunctional probes-primers to amplify a *tcdB* fragment. Samples in Tris-EDTA buffer are heat-treated; the lysate is used directly to perform the test. The system can accommodate a maximum of 94 samples and has an assay time of 91 min. When compared to another FDA-cleared assay, the Meridian Illumigene Asssay, the Simplexa *C. difficile* Assay had a sensitivity of 98% and a specificity of 100%; the concordance between the two systems was 98.7%[99].

AmpliVue *C. difficile* assay: The AmpliVue *C. difficile* assay uses helicase-dependent, isothermal amplification of a highly conserved 83-bp fragment of the *tcdA* gene. The assay includes a disposable detection device that allows for visual evaluation of amplification results. The AmpliVue system can perform a maximum of 24 assays, and has a total running time of 73 min. The AmpliVue assay had a sensitivity of 96% and specificity of 100% when compared to the FDA-cleared assay Meridian Illumigene Assay[99]. Compared to TC, the sensitivity and specificity were 91% and 89%, respectively[100].

Illumigene *C. difficile* assay: The Illumigene *C. difficile* assay uses loop-mediated isothermal DNA amplification technology to target a partial DNA conserved region of *tcdA* common to A+B+ and A-B- strains. The total time of analysis is 68 min and the maximum number of samples per run is 10[99]. When compared to TC, the sensitivity and specificity of the Illumigene assay were 100%[101].

BD GeneOhm Cdiff assay: The BD GeneOhm Cdiff assay (BD Diagnostics, San Diego, CA) amplifies the *tcdB* gene from stool samples with *C. difficile,* which is detected and analyzed with a SmartCycler instrument (Cepheid, Sunnyvale, CA)[102]. The performance of the BD GeneOhm Cdiff PCR assay has been compared to the Tox A/B II ELISA and a two-step method composed of the C. Diff Chek-60 GDH antigen assay followed by cytotoxin neutralization on 105 true positive samples. The detection rate was 66.7% for the Tox A/B II ELISA assay, 82.9% for the 2-step method, and 91.4% for the BD GeneOhm Cdiff PCR assay. The overall concordance between the BD GeneOhm Cdiff PCR assay and the Tox A/B II ELISA was 91.3%, while the concordance between the BD GeneOhm Cdiff and the two-step method was 93%. The BD GeneOhm Cdiff PCR and the two-step algorithm had similar performance but were more sensitive than Tox A/B II. Compared to the two-step algorithm, BD GeneOhm Cdiff PCR is faster but almost five times more expensive; BD GeneOhm Cdiff PCR is also six times more expensive than the Tox A/B assay[102]. When compared to TC, BD GeneOhm Cdiff PCR has a sensitivity of 89.6% and a specificity of 96.7%[103].

ProGastro Cd assay: ProGastro Cd assay (Prodesse, Waukesha, WI) is a Taqman real-time PCR assay that detects the *tcdB* gene. Amplification is performed on the Cepheid SmartCycler II (Sunnyvale, CA). Stool samples are processed to obtain genetic material using the NucliSENS easy MAG platform (bioMérieux, Inc., Durham, NC)[104].

There was a 95.7% agreement between TC and the ProGastro Cd assay. When comparted to TC, the ProGastro Cd assay had a sensitivity that ranged from 77.3% to 100% and a specificity between 99.2% and 93.4%[103,104]. A two-step algorithm, with the GDH-based C. Diff Quik Chek Complete as step 1 and the ProGastro Cd assay as step 2, yielded an estimated sensitivity of 97.9% and a specificity of 95.4%.

Multiplex platforms: There are two types of multiplex platforms: the first type includes the so-called "syndromic" platforms*, i.e.,* a platform to detect pathogens associated with a particular symptom. In this case, a variety of syndromic platforms test for the main causative agents of diarrhea, independently of the kind of microorganism (bacteria, viruses or protozoa). The second type are pathogen class specific multiplex molecular assays[105].

Currently, there are two FDA-cleared syndromic multiplex assays that include the dectection of *C. difficile*: Luminex xTAG GPP (Luminex Molecular

Table 3 Summary of non Food and Drug Administration-approved multiplex assays that detect *Clostridium difficile*

Assay	Company	Pathogens detected	Technology	Ref.
Gastrofinder Smart 17 Fast	PathoFinder	9 bacteria, 4 viruses and 4 parasites	Multiplex Real-time PCR	[129]
EasyScreen Enteric assays	Genetic Signature	7 bacteria, 8 viruses and 5 parasites	3base Technology	[130]
RIDA®GENE	R-BioPharma AG	11 bacteria, 4 viruses and 4 parasites	Multiplex Real-time PCR	[131]
FTD® Bacterial Gastroenteritis	Fast-Track Diagnostics	9 bacteria, 5 viruses and 3 parasites	Multiplex Real-time PCR	[132]
CLART EnteroBac panel	Genomica	19 bacteria	Low-density microarray	[133]
Faecal Bacteria	AusDiagnostics	8 bacteria, 4 viruses and 3 parasites	Multiplex Tandem PCR technology	[134]
Seeplex Diarrhea ACE	Seegene	10 bacteria and 4 viruses	Dual priming oligonucleotide technology	[135]

Diagnostics Inc., Toronto, Canada) and BioFire FilmArray GI Panel (BioFire Diagnostics, Salt Lake City, UT)[106]. In addition, there is a non-FDA cleared, but "Conformité Européene" (CE) marked multiplex assay, the Gastrofinder Smart 17 Fast (PathoFinder, Maastricht, The Netherlands)[105]. There are six additional commercially available platforms that include *C. difficile* among their targets, but none of these platforms are FDA cleared or CE marked[105].

Luminex xTAG pathogen panel: This assay simultaneously detects *Salmonella* sp., *Shigella* sp., Shiga toxin-producing *E. coli* (STEC) stx1/stx2, *Vibrio cholerae*, *Yersinia enterocolitica*, *C. difficile* toxin A/B, *Campylobacter* sp., *E. coli* O157, Enterotoxigenic *E. coli* (ETEC) LT/ST, adenovirus 40/41, rotavirus A, norovirus GI/GII, *Giardia lamblia*, *Cryptosporidium* sp. and *Entamoeba histolytica*[107]. The assay performs a multiplex reverse transcriptase PCR, using tagged and biotinylated primers. Amplicons are detected by hybridization to the pathogen-specific complementary antitag sequence coupled to specific beads and binding of the streptavidin-phycoerythrin reporter to the biotinylated primers[106,107].

The performance of the Luminex xTAG panel was evaluated with 185 stool samples from 176 patients. In 11% of the samples, multiple pathogens, including ETEC, *G. lamblia*, norovirus, *Shigella* sp., *Campylobacter* sp., *Salmonella* sp., adenovirus, and *C. difficile,* were detected[107]. There was a 100% sensitivity, specificity, PPV, and NPV. Another study evaluated the assay with a total of 254 clinical specimens[108]. Depending on the target organism, the sensitivity ranged from 90% to 100% with an overall sensitivity of 94.5% and a specificity of 99.1%. With respect to *C. difficile*, the sensitivity, specificity, PPV, and NPV were 91%, 100%, 100%, and 99%, respectively.

In a site-specific clinical evaluation, the Luminex xTAG Gastrointestinal assay showed a sensitivity of 98.7% and a specificity of 99.8%. *C. difficile*, *Salmonella* spp., and *Cryptosporidium* spp. accounted for 67% of the targets detected. Specifically, *C. difficile* was detected in 23 samples, all of which were confirmed with the Cepheid Xpert *C. difficile* assay. Furthermore, the multiplex detection led to savings in the hospital ward as compared to traditional methods[109].

FilmArray GI panel: The FilmArray uses nested multiplex PCR that is executed in two stages. First, the FilmArray performs a reverse transcription PCR. Next the diluted products are combined with a fluorescent double-stranded DNA-binding dye. This solution is aliquoted into an array with wells that contain primers to amplify internal sequences of the first product, so that in each well an individually nested PCR is performed. Results are obtained after fluorescence analysis[106,110].

The assay detects *Campylobacter* (*C. jejuni*, *C. coli* and *C. upsaliensis*), *C. difficile*, *Plesiomonas shigelloides*, *Salmonella*, *Y. enterocolitica*, *Vibrio* (*V. parahaemolyticus*, *V. vulnificus,* and *V.cholerae*), *E. coli* O157, Enteroaggregative *E. coli*, Enteropathogenic *E. coli*, ETEC, STEC, *Shigella*/Enteroinvasive *E. coli*, Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, Sapovirus (Ⅰ, Ⅱ, Ⅳ, and Ⅴ), *Cryptosporidium*, *Cyclospora cayetanensis*, *E. histolytica* and *G. lamblia*. For the identification of *C. difficile*, the FilmArray GI Panel targets both *tcdA* and *tcdB*[111].

In a study that evaluated the BioFire FilmArray GI Panel, *C. difficile* was the most frequently detected pathogen (83 out of 378 samples, 22%). The sensitivity of the BioFire FilmArray Gastrointestinal Panel to detect *C. difficile* was 95% and the specificity 99% using the Illumigene as a reference. In 91 episodes for which specific testing for *C. difficile* was ordered, 42 episodes (46%) were *C. difficile* positive according to standard testing and 40 (44%) according to the FilmArray GI Panel in 40 (44%)[112].

In a multicenter evaluation, the BioFire FilmArray GI Panel detected *C. difficile* in 204 out of 832 positive samples, contrary to 165 positive samples detected by the comparator (PCR of toxin A and B genes), resulting in a positive percent agreement of 98.8% and negative percent agreement of 97.1%[111].

Non-FDA approved assays

There are seven non FDA-approved assays on the market that include *C. difficile* among their targets (Table 3). The majority of these assays, employ real-time PCR technology. To our knowledge, only three of them have been evaluated regarding the sensitivity and specificity at detection *C. difficile*.

The RIDA® GENE CD PCR assay (R-Biopharm AG, Darmstadt, Germany), when compared to TC as a reference, had a sensitivity of 98.1%, a specificity

of 100%, a PPV of 100%, and a NPV of 98.1%[113]. Ylisiurua *et al*[113] found that the RIDA® GENE CD PCR assay outperforms competing molecular *C. difficile* assays, such as GeneOhm™ Cdiff assay (Becton Dickinson) and Xpert® C. difficile test (Cepheid).

The EasyScreen Enteric Bacterial Detection Kit is a multiplex assay with calculated analytical sensitivities that range from 2.5 to 12.5 copies of targeted pathogen, free of cross-reactivity to non-target microorganisms. For *C. difficile*, the calculated sensitivity was 100% and the specificity 81.2%. The EasyScreen assay correctly identified all *C. difficile*-containing samples (12 out of 18), including the ribotype 027 and 078 strains (1 of each)[114].

The Seeplex® Diarrhea ACE is another multiplex molecular assay that was compared to BD GeneOhm, with TC as a reference. There was a positivity rate of 35.4% (86/243). The concordance rate between the BD GeneOhm and Seeplex® Diarrhea ACE assay was 96% (234/243) with no significant differences between them. The sensitivity, specificity, PPV, and the NPV of the Seeplex® Diarrhea ACE assay were 90.0% (63/70), 97.1% (168/173), 92.6% (38/43), and 96.0% (168/175), respectively[115].

BIOMARKERS

CDI is accompanied by intestinal inflammation. Inflammation biomarkers include cytokines, calprotectin, and fecal lactoferrin[116]. These biomarkers are not disease specific, but may be indicators of severity[117]. For example, fecal lactoferrin has been evaluated in both infectious diarrhea and inflammatory bowel disease. Fecal lactoferrin, blood biomarkers, white blood cell count and low serum albumin level, were significantly associated with severe CDI and stool toxin[116]. Furthermore, age, Charlson co-morbidity index, intensive care treatment, increased peripheral white blood cell count, elevated lactoferrin, decreased albumin, and elevated creatinine were significantly associated with death within 100 d of CDI diagnosis[118].

Calprotectin is a protein found in the cytoplasm of neutrophils and can be detected in stool in cases of intestinal inflammation, such as in inflammatory bowel disease and infectious diarrhea[117,119]. A large proportion of individuals with nosocomial diarrhea (diagnosed by PCR) have elevated levels of calprotectin[120]. High fecal levels of calprotectin have been associated with *C. difficile* strain 027[121] and complicated/recurrent CDI in a cohort of older adults[119]. Though elevated stool calprotectin has a low sensitivity as a diagnostic test (38.5%), its specificity for complicate or recurrent CDI was 91.9%, and thus provides valuable information for adequate treatment decision-making[119].

So far, there is no specific biomarker that detects CDI or any other pathogenic agent of clinically significant diarrhea. Anikst *et al*[122] suggests the implementation of measures to avoid unnecessary testing for *C. difficile* in order to diminish CDI overdiagnosis. The identification of such a biomarker, will be helpful to improve CDI diagnosis.

CONCLUSION

C. difficile has a worldwide distribution; its toxigenic strains are responsible for CDI. Despite increasing knowledge on risk factors that favor CDI and measures to reduce propagation, there is an increase in the prevalence of CDI in many countries[123-127]. One of the challenges at managing of CDI is the initial diagnosis of the disease. To date, there is no single test that accurately and rapidly diagnoses CDI. Multistep testing is recommended for a diagnosis with acceptable sensitivity and specificity. The inclusion of NAATs in the diagnostic algorithm combines high sensitivity with a short turnaround time. However, test results should be interpreted with caution and should consider clinical suspicion, the presence of risk factors, and a correct interpretation of test results. A better understanding of the pathogenesis of *C. difficile* will help both physicians and laboratories to develop the best strategy to overcome current issues with CDI diagnosis.

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ISSN 1007-9327
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