Laboratory diagnosis of *Clostridium difficile* disease

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The laboratory diagnosis of *Clostridium difficile*-associated disease (CDAD) is based on culture and toxin detection in fecal specimens. Culture is performed on a commercially available selective media. *C. difficile* colony morphology is typical when viewed under a dissecting microscope. Definitive identification is best obtained by gas liquid chromatography. Culture is very sensitive but, when used alone without toxin testing, it leads to low specificity and misdiagnosis of CDAD when high rates of asymptomatic carriage exist. Toxin detection by a tissue culture cytotoxin assay followed by neutralisation with specific antiserum is often considered the standard. However, this approach lacks sensitivity and has not detected up to 30% of patients with confirmed CDAD. Multiple enzyme immunoassays (EIAs) have been introduced by various manufacturers for the detection of toxin A alone or for both toxins A and B. Some of these are designed to give results in less than 1 h. Comparative studies of EIA kits reported that the sensitivity and specificity are slightly lower than cytotoxin assays. Toxigenic culture tests *C. difficile* isolates for toxin production: colonies isolated on selective media are tested for in-vitro toxin production either by a cytotoxicity assay or by direct EIA. It has higher sensitivity than the cytotoxicity assay and equivalent specificity. In the routine laboratory, culture and toxin detection should be performed on every specimen and, in culture-positive and fecal toxin-negative cases, toxigenic cultures should be performed on isolated colonies.

Keywords *Clostridium difficile*, diagnosis

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INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic sporulating bacillus which has been known since 1978 to cause a severe disease of the colon called pseudomembranous colitis. One of the most remarkable characteristics of this disease is that, in almost all cases, it occurs following antibiotic therapy. The pathogenesis of *C. difficile*-associated disease (CDAD) is very particular and allows a better understanding of the circumstances in which the clinician should make a diagnosis (Figure 1).

PATHOGENESIS OF CDAD

The very first condition to induce pathology is a disturbance of the normal intestinal flora. The gut flora act as a colonisation barrier which, in a normal state, protects against *C. difficile*. This barrier is compromised when the flora is disturbed. The main factor able to induce such a disturbance is antibiotic therapy. Almost all compounds have been implicated, in particular the broad-spectrum cephalosporins and all molecules active on anaerobic flora.

There are several other circumstances which have been reported to induce such a disturbance, such as cancer chemotherapy or antacid treatment, but these account for a minority of cases. Finally, neonates who do not yet have constituted flora, are very often colonised by *C. difficile* but, for several poorly understood reasons, they remain asymptomatic in most cases.

Colonisation by *C. difficile* constitutes the second step of the disease. Intestinal carriage of *C. difficile* is estimated to be very low, between 1 and 2% and, hence, colonisation from an endogenous source is relatively rare. The main concern with this bacteria is that, once hospitalised patients get diarrhea, they very rapidly contaminate their environment with spores which are very resistant and may persist for months, thus creating the potential for a hospital outbreak. At present, *C. difficile* is considered to be the most common cause of diarrhea in hospitalised patients [1].

In the case of colonisation, *C. difficile* usually produces two toxins called A and B which are the main virulence factors. Not all strains produce these toxins. One of the most interesting features of CDAD is that there is a very wide range of clinical presentations; even in the case of toxin production, a patient may remain asymptomatic, or exhibit only mild diarrhea, whereas others may exhibit severe pseudomembranous colitis or even fulminant life-threatening colitis.

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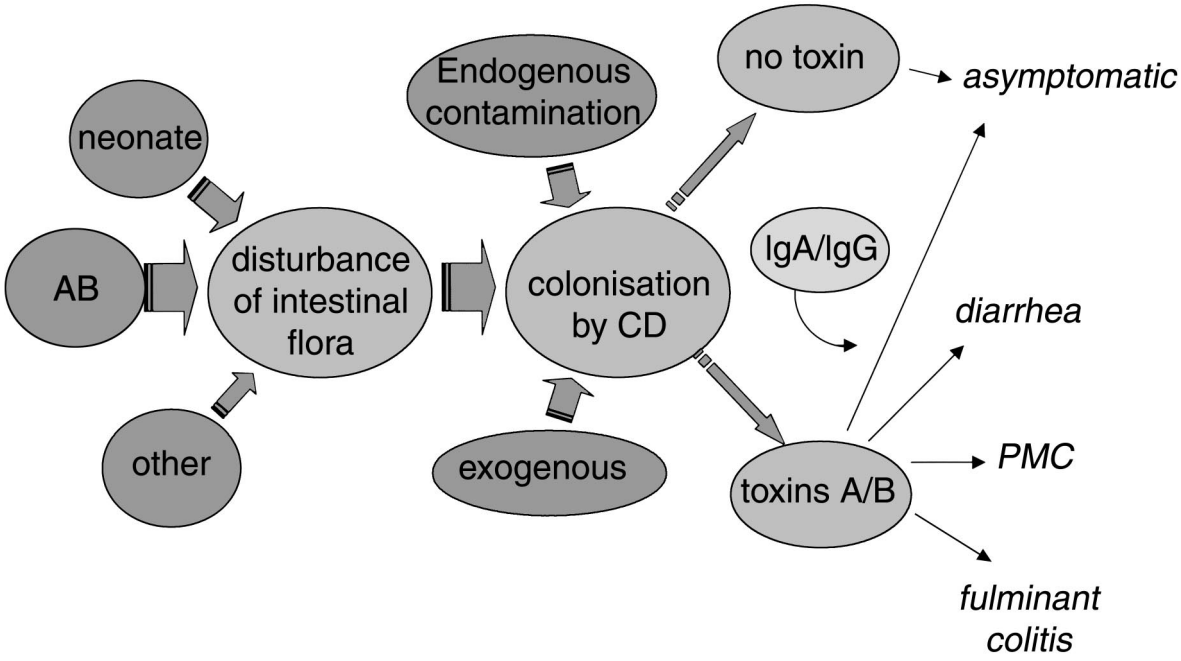

Figure 1 Pathogenesis of *Clostridium difficile*-associated diarrhea.

The factors that influence the severity of the symptoms are now better understood. For instance, the age of the patient is an important factor; most of the severe cases are seen in patients over 65 or 70 years. However, as demonstrated in several recent publications, what now appears to be the main factor is the level of the immune response in terms of circulating IgG or local IgA against toxin A. Patients with severe symptoms have significantly lower serum- and feces-specific antibody levels than those with milder symptoms [2]. Moreover, a serum antibody response to toxin A during an initial episode of CDAD is associated with protection against recurrence [3]. It has also been shown that, after colonisation of a patient by *C. difficile*, there is an association between a systemic response to toxin A, as evidenced by increased serum levels of IgG antibody against toxin A, and asymptomatic carriage of *C. difficile* [4]. Therefore, given this scheme, the main indication – but not the sole one – for a bacteriological diagnosis of CDAD is diarrhea occuring in elderly, hospitalised patients with a history of antibiotic therapy. Several authors have advocated testing only for *C. difficile* in cases of diarrhea occurring in patients hospitalised for more than 3 days [5,6].

The clinical diagnosis of CDAD can be made by rectoscopy or colonoscopy when classical pseudomembranes are seen. A negative finding, however, does not rule out the diagnosis since endoscopic diagnosis has a poor sensitivity. When a clinician suspects CDAD, a laboratory confirmation should be requested and a fecal specimen submitted.

LABORATORY DIAGNOSIS

A laboratory diagnosis of CDAD is based on the isolation of the pathogen in stool specimens by culture and by toxin detection, which can be performed either by detection of a cytopathic effect of a stool filtrate on cell lines or by a direct enzyme immunoassay (EIA). The cytotoxin detection is advocated by many authors, especially in the USA, as the standard diagnosis but both culture and toxin detection are necessary to achieve an optimal result.

Other indirect tests have been proposed previously, such as direct gas liquid chromatography (GLC) on stool specimens, latex agglutination or computed tomography scan, but these approaches do not reach sufficient sensitivity and specificity to be accepted unless they are used in addition to the other tests.

For an optimal bacteriological diagnosis, only liquid stools should be accepted, except in the case of an epidemiological investigation. Due to a rapid loss of cytotoxin activity, only fresh specimens should be processed and they should be stored at 4 °C or less, in case tests cannot be performed rapidly. Brazier reported complete inactivation of cytotoxin in about 20% of samples sent through the post [7]. On the other hand, cultures of *C. difficile* remain unaffected by ambient storage due to sporulation. Repeated samples within 7 days of the initial request seem to give little useful information [8].

CULTURE

Just after the discovery of the pathogenic role of *C. difficile*, George et al. proposed a selective agar plate called CCFA (cycloserine cefoxitin fructose agar) for the isolation from stool specimens [9]. This medium is still used in most laboratories. The selective agents are cycloserine at a concentration of 500 mg/L and cefoxitin at 16 mg/L. These concentrations were reduced to 250 and 8 mg/L, respectively, in some studies [10]. The original formulation included egg-yolk, which may be replaced by blood. It is commercially available from several companies. Stools are directly inoculated and incubated in an anaerobic atmosphere for 48 h. Anaerobic preincubation of the plate may improve the recovery rate as well [11]. In our experience, the use of an Anoxomat system (Mart Microbiology NV, Lichtenvoorde, Netherlands), which allows an anaerobic atmosphere to be obtained in the jar within 1 min, allows the plates to be read after only a 24 h incubation.

Several modifications have been proposed to enhance the sensitivity of the culture. Most are aimed at recovering more spores and are more dedicated to epidemiological studies and environmental cultures. Addition of pure sodium taurocholate or cholate at a concentration of 1 g/L allows a better spore germination [12] and is suitable for environmental cultures or in cases of negative culture with positive fecal cytotoxin (see below). The sodium salt of cholic acid is just as effective as pure taurocholate in stimulating spore germination but is much less expensive [7]. Pretreatment of stools with ethanol-shock (equal volumes of ethanol and feces mixed for 1 h before inoculation) has also been shown to increase the sensitivity of culture [13].

It is worth saying that, in the vast majority of cases, the use of a standard CCFA medium with a standard inoculation procedure is quite satisfactory for diagnosis. Culture is the most sensitive method but it is not very specific due to the possibility of isolating non-toxigenic isolates. It is slow but allows strains to be tested for toxigenicity; it is also the only way to carry out epidemiological investigations.

Colonies of *C. difficile* are easily recognised on culture plates due to their typical morphology (ground glass appearance) when observed with binoculars. A yellow-green or chartreuse fluorescence under ultraviolet illumination is another characteristic of the colonies but this may vary with the medium used. The typical odor (horse manure) is also an aid to identification. GLC of an agar plug around a suspected colony is the best and easiest method for confirming identification. *C. difficile* displays a typical GLC profile with large amounts of butyric and iso-caproic acids. Such devices, however, are not available in many laboratories. Individual biochemical tests or anaerobic panels may also be used, as well as a somatic antigen latex kit. Cross-reactions, however, have been documented with this latter reagent. A direct EIA for toxin A may also be used but then, of course, it will only recognise toxigenic isolates. Production of proline-aminopeptidase by a disc test has been proposed recently as a means of rapid identification of *C. difficile* when it is used in conjunction with the typical morphology of the colonies [14].

TOXIN DETECTION

Cytotoxin detection is often considered as the standard for the diagnosis of *C. difficile* infections. This method consists of inoculating a filtrate of a stool suspension into a cell culture and observing a cytopathic effect as a consequence of disruption of the cell cytoskeleton; which results in cell rounding in many cell lines. The effect is mainly due to toxin B, which is 1000 times more cytotoxic than toxin A.

Almost all cell lines commonly used in clinical microbiology laboratories can be used to detect fecal cytotoxin: Vero, Hep2, fibroblast, CHO or HeLa cells are the most common. Vero cell lines are considered by many to be the most sensitive. A suspension of 1 : 5 of the fecal sample is made in phosphate-buffered saline and, after centrifugation, the supernatant is filtered through a 0.2-μM filter. The filtrate is then inoculated on the cell monolayer and the presence or absence of the cytotoxic effect is observed after 24 and 48 h. In some of the most severe clinical cases, typical rounding may be observed after 4–6 h. Confirmation of the specificity is obtained by repeating the test with the addition of a specific antiserum directed against *C. difficile* or against *C. sordellii*, which shares the same antigens.

The method has many advantages. It is sensitive and specific, and can detect other clostridial toxins. On the other hand, it is relatively slow in comparison to EIA. In addition, non-specific cytopathic effect (not neutralised by antiserum) may be observed in about 2% of the cases, rendering any interpretation impossible. The necessity of maintaining cell lines, which is both time-consuming and expensive, especially if only a small number of specimens are processed, is another drawback.

For the last 10 years, there have been many EIAs on the commercial market. Establishment of a complete list is difficult since not all are available in every country. Most use monoclonal antitoxin A antibodies, whereas a few are designed to detect both toxins. Finally, in one of the latest kits, the detection of toxin A is coupled with the detection of a glutamate dehydrogenase, a *C. difficile*-specific enzyme found in toxigenic as well as in non-toxigenic isolates. The reason why kits detecting both toxins have been developed is mainly because some isolates from clinical cases have been shown to produce only toxin B [15]. In our experience, such strains are very rarely observed. In England, however, it has been found to account for 3% of the strains referred to the reference laboratory

for typing [16], and outbreaks have been described in Canada [17]. Some kits are presented as individual panels, others as 96-well microplates. One of the main advantages of these kits is their rapidity, since results can be obtained within 20 min.

There have been numerous publications regarding the performances of the different kits. When compared to fecal cytotoxin detection on cell lines, the different EIAs show a slightly lower sensitivity. In our hands, cytotoxicity could be detected in a 1/64 dilution of a positive fecal sample, although only at half-dilution for most of the EIAs (unpublished results). The same observations have been made in clinical studies [18–23].

In several studies, toxigenic culture and detection of toxigenicity have been demonstrated as the most sensitive and specific technique for the diagnosis of CDAD [18,22,24,25]. In this approach, culture of fecal specimens on CCFA is followed by in vitro determination of the toxigenicity of the positive colonies by the cytotoxin test or by EIA. Most comparisons of EIA kits against toxigenic culture have demonstrated lower performances of EIAs.

One of the most recent kits, Triage *C. difficile* panel (Biosite Diagnostics, San Diego, CA, USA), combines the detection of toxin A and of glutamate dehydrogenase (GDH), an enzyme which is produced by *C. difficile*. Although the sensitivity of toxin A detection is within the same range of other kits (79.4%), the addition of the GDH test allows an excellent negative predictive value of an infection by *C. difficile* to be obtained [26,27]. A negative result for both tests at the same time means that the presence of a toxigenic strain of *C. difficile* can be excluded with a reliability of 99.6% [27]. It may be used as a rapid screening test to decide which stools need to be processed further.

Although less sensitive, the EIAs provide same-day results and can be used as a screening test. They may be useful in laboratories without tissue-culture facilities. They should always be combined with culture and, when negative with a positive culture, should be repeated by testing the strain isolated on the plate.

MOLECULAR METHODS

Molecular methods for the diagnosis of CDAD have been studied far less than those used to diagnose other infectious diseases. This is probably due to the relatively satisfactory results obtained with classical methods as described above, as well as to the difficulty in applying such methods to fecal specimens known to interfere with amplification procedures.

Several methods based on the PCR for amplifying part of the toxin A gene have been effective in distinguishing toxigenic from non-toxigenic isolates, but these approaches seem to have few advantages over the routine laboratory methods.

Direct detection of *C. difficile* genes in fecal specimens have been tested as well. Oligonucleotide probes designed to detect toxin B have been used by Green et al. [28] with sensitivity and specificity in the same range as EIAs. PCR using sets of primers designed to detect toxin A or B have been tested on stools by several authors [29–32]. The procedures usually comprise a preliminary step to avoid inhibitory substances. So far, they have been tested in small series of specimens and the results have not shown any significant improvement when compared with the classic methods.

CONCLUSIONS

During the last 15 years, our laboratory has received a total of 20 698 diarrheal stools from patients over 10 years old, for the diagnosis of CDAD. The results of both fecal culture and cytotoxin assay were negative in 17 731 cases (86%). Culture was positive in 2622 cases (13%) but less than half of these positive cultures (1,149) also had a positive fecal cytotoxin. When testing culture-positive, cytotoxin-negative isolates, 852 (58%) were shown to be toxigenic strains in vitro. This means that, performing the cytotoxin assay alone, 852 stools (4.1%) with toxigenic strains would have been overlooked. In our viewpoint, these observations justify the following scheme for the routine bacteriological diagnosis of CDAD (Table 1); culture and toxin detection (by cytotoxicity or by EIA) should be performed on every specimen. When both tests are negative

Table 1 Proposed scheme for the bacteriological diagnosis of CDAD

Initial tests on stools			
Culture	**Fecal toxin**	**Additional test**	**Conclusion**
−	−		No CDAD
+	+		CDAD
+	−	Strain in vitro toxigenicity	
		+	Probable CDAD
		−	Carriage of non-toxigenic *C. difficile*
−	+	Repeated culture on CCFA + taurocholate	Probable CDAD

(as in 87% of the cases), a diagnosis of CDAD is excluded. If both are positive, a diagnosis of CDAD necessitates treatment and the implementation of hospital hygiene prevention measures.

When culture is positive but toxin remains negative, a direct EIA test should be performed on several colonies picked up directly on the culture plate and, hence, within 30 min it is determined whether the strain is toxigenic or not. If the test is negative, treatment for CDAD is not necessary. If the test is positive, CDAD is very likely and the same measures as for a direct positive test should be adopted. On the very few occasions where culture is negative but there is a positive cytotoxin test (0.2%), a control specimen is usually requested and the culture repeated with a CCFA including taurocholate; a positive result is usually obtained.

One could object that low carriage rates of toxigenic strains with no detectable level of toxin might indicate carriage rather than an infection. We believe, however, that patients with diarrhea who are carriers of a toxigenic strain are likely to contamine the environment; this reason alone justifies performing the culture.

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