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## Editorial

### Laboratory tests for diagnosis of *Clostridium difficile* infection: Past, present, and future

### Pruebas de laboratorio para el diagnóstico de la infección por *Clostridium difficile*: pasado, presente y futuro

Luis Alcalá<sup>a,b,c,\*</sup>

<sup>a</sup> Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria del Hospital General Universitario Gregorio Marañón, Madrid, Spain

<sup>b</sup> Red Española para la Investigación en Patología Infecciosa (REIPI), Madrid, Spain

<sup>c</sup> Centro de Investigación Biomédica en Red Enfermedades Respiratorias (CIBERES), Madrid, Spain

*Clostridium difficile* was first isolated in 1935 in the normal flora of newborns.<sup>1</sup> Since 1978, it has been reported to be a cause of antimicrobial-associated diarrhea, colitis, and pseudomembranous colitis.<sup>2</sup> During the late 1970s and early 1980s, several studies concluded that two toxins, toxin A (enterotoxin) and toxin B (cytotoxin), were responsible for the characteristic symptoms of *C. difficile* infection (CDI).<sup>3</sup> Subsequent investigations showed that not all strains of *C. difficile* expressed these toxins and that toxin-producing strains possessed a 19.6-kb pathogenicity locus containing the *tcdA* and *tcdB* genes, which encoded toxin A (308 kDa) and toxin B (269 kDa), respectively. Therefore, as only toxigenic strains can cause disease, diagnosis of CDI should be based on the detection of toxins instead of only on the detection of the bacterium.

The first useful method for the diagnosis of CDI was the cell cytotoxicity assay, which was developed at the end of the 1970s.<sup>4</sup> This method was based on the detection of cytotoxic activity in stool specimens, which was observed by rounding of cells in tissue culture and neutralization of the activity by *C. difficile* or *C. sordellii* antitoxin. The high correlation between the results of this technique and the presence of disease led it to be considered the gold standard for the diagnosis of CDI for many years. During the same period, the first specific and effective selective media containing cycloserine, cefoxitin, fructose, and egg yolk (CCFE) were being developed.<sup>5</sup> Subsequent modification of these media—replacement of egg yolk by blood and addition or modification of antimicrobials—improved the yield of *C. difficile* culture.

During the 1980s, the development of enzyme immunoassays (EIAs) capable of detecting *C. difficile* toxins was a considerable advance in the laboratory diagnosis of CDI.<sup>6</sup> The ease of use, speed (minutes to hours), and relatively low cost of these tests compared with the cytotoxicity assay and culture led to their widespread

use in diagnostic laboratories. The first EIAs were based on wells in which results were displayed as a color change detected visually or by spectrophotometry and could be obtained in several hours. Subsequent EIA designs based in immunochromatography enabled results to be read visually from a membrane and provided a diagnosis easier and sooner (<1 h), although with a slightly lower sensitivity than that achieved from well-based EIAs. Because toxin A is more stable and easier to purify than toxin B, the first EIAs only detected this antigen. However, increased knowledge of the pathogenic strains toxin A– and toxin B+ meant that these techniques were replaced by EIAs that detected both toxins. The first comparisons with the cytotoxicity assay as the gold standard showed that most EIAs had sensitivities greater than 80% and specificities near 100%.<sup>7</sup> In an attempt to further enhance ease of use and reduce hands-on time to only 3 min, a latex agglutination test detecting toxin A was developed in the mid-1980s and soon became very popular. When compared with the cytotoxicity assay, latex agglutination was shown to be very sensitive, although not very specific. Several studies showed that this lack of specificity was due to this test really detected an enzyme—glutamate dehydrogenase (GDH)—that was characteristic of both toxigenic and non-toxigenic *C. difficile*. An explanation for this unexpected finding was that the antiserum containing toxin A used in the preparation of this technique was contaminated with GDH. Consequently, the procedure fell into disuse for many years in most diagnostic laboratories. Another important advance in the diagnosis of CDI during the 1980s was toxigenic culture, which involved the culture of *C. difficile* strains from stool specimens and detection of toxins directly from isolates using cytotoxicity assay or toxins EIAs. In 1982, Chang and Gorbach<sup>8</sup> compared this procedure with the cytotoxicity assay and concluded that toxigenic culture was more sensitive and that both techniques should be used together to ensure accurate diagnosis of CDI. However, this important recommendation had no impact on diagnostic laboratory procedures for many years, possibly owing to the complexity of the procedure, which can take at least 24–48 h and requires a virology laboratory

\* Corresponding author.

E-mail address: luisalcala@efd.net

to supply cells when cytotoxicity assay is used to confirm toxin B on isolates.

During the 1990s some diagnostic tests were improved by automation, as was the case with some EIAs,<sup>9</sup> and more sensitive and specific tests were designed. The most important development during this decade was probably polymerase chain reaction (PCR) for the detection of the toxin B gene in stools in 1993.<sup>10</sup> Although the validity of the technique was very good, the use of PCR for diagnosis of CDI was not generalized until two decades later.

Clearly, the epidemic of CDI first described in 2002 in Quebec, Canada,<sup>11</sup> and later extended to many countries from all over the world, marked the evolution of CDI diagnosis during the beginning of this century. Pepin et al. reported the emergence of an epidemic strain, NAP1/027, which was characterized by an increased case-fatality rate, especially in patients treated with metronidazole instead of vancomycin. This finding increased the interest on CDI and led to improved testing, new antimicrobials, and the availability of guidelines for the management of CDI.<sup>12</sup> The need for an accurate test that enabled rapid diagnosis of CDI became clear, especially when EIAs were shown to be insensitive and not very specific when compared with toxigenic culture, a new good standard more sensitive than the cytotoxicity assay.<sup>7</sup> Industry interest in the diagnosis of CDI led to a re-examination of the work of Kato et al.<sup>13</sup> and the subsequent development of automated techniques based on real-time PCR or LAMP (loop-mediated isothermal amplification) technology for the detection of *C. difficile* toxin genes. Tenover et al.<sup>14</sup> reviewed the 4 commercial amplification tests approved by the FDA at the time. Three were real-time PCR assays capable of detecting at least toxin B gene, while the remaining used LAMP for the detection of a conserved part of toxin A. Most of the tests had a turnaround time of less than 2 h with minimal hands-on time (a few minutes in some cases). Sensitivity and specificity were up to 95% and 99%, respectively, when compared with toxigenic culture. However, as molecular techniques detect genes and not toxins, clinicians must be aware of a positive result and differentiate colonization from CDI. In addition, the relatively high cost of these tests prevented their use in the daily routine of most laboratories.

The absence of any technique that, used as a stand-alone, is cost-effectiveness for the rapid diagnosis of CDI has led to some authors to evaluate multistep algorithms based on GDH detection as the screening test followed by confirmatory tests as toxin A and B EIAs, cytotoxicity assay, toxigenic culture, or molecular assays. In a recent work published in *Enfermedades Infecciosas y Microbiología Clínica*, Orellana-Miguel et al.<sup>15</sup> evaluated the performance of an algorithm based on initial screening using an immunochromatography (ICT) that detects both GDH and toxins A and B (TAB) (Techlab® C. diff Quik Chek Complete, Inverness Medical Innovations, Inc., Princeton, New Jersey, USA) and a toxigenic culture as a confirmatory test in those specimens yielding a GDH+/TAB– result. The gold standard in this study consisted of toxigenic culture using CLO agar (bioMérieux, Marcy l'Etoile, France) without ethanol pre-treatment. In the case of a negative toxigenic culture and a GDH+ result, a second toxigenic culture was performed after pre-treatment with ethanol. Sensitivity, specificity, and positive and negative predictive values (%) for the tests evaluated were as follows: GDH alone (100, 96.1, 61.7, and 100), TAB alone (56.9, 99.9, 97.0, and 97.3), and proposed algorithm (100, 99.9, 98.3, and 100). The authors concluded that the algorithm, that applied the toxigenic culture as a confirmatory test in only 6.2% of the specimens, can diagnose CDI very accurately with validity values near 100%.

The sensitivity of GDH detection, that determines to a large extent the overall sensitivity of GDH-based algorithms, is variable in literature (70–100%).<sup>16,17</sup> In our own institution, that is using the ICT evaluated by Orellana-Miguel et al. from two years ago, the sensitivity of GDH detection is about 87%. These differences among

values could be explained by several factors such as the different commercial tests used or the prevalent ribotypes present in each study, as outlined by Orellana-Miguel et al.<sup>15</sup> In any case, the sensitivity of GDH detection should be monitored at least periodically using toxigenic culture in order to ensure correct performance of CDI diagnosis.

The confirmatory test used after GDH detection is essential to validate positive results of this initial technique. Algorithm evaluated by Orellana-Miguel et al.<sup>15</sup> included toxin EIA detection as an intermediate confirmatory test. Inclusion of this test is a simple, cheap and rapid procedure to confirm positive GDH results because its positive predictive value increases nearly to 100% when evaluated together with GDH results. Moreover, simultaneous detection of GDH and toxins A and B could accurately diagnose about half of all CDI episodes and thus confirm the diagnosis on the day of specimen processing and eliminate the need for other confirmatory tests in these specimens. From the point of view of patient care, a final confirmatory test, with or without an intermediate test, must ideally be rapid, sensitive and specific. Consequently, the cytotoxicity assay would be excluded because they are not sensitive. Toxigenic culture, although both sensitive and specific, takes a median of two days from specimen processing to results. For this reason, an increasing number of microbiologists and international guidelines<sup>18</sup> (<http://www.asm.org/images/pdf/Clinical/clostridiumdifficile9-21.pdf>) now consider the optimal cost-effectiveness algorithm to be a combination of GDH detection as the screening test and a molecular technique as the final confirmatory test. Results obtained by Orellana-Miguel et al. and other authors including us confirm that it would be necessary to test with molecular methods about 15% of the initial screened specimens. Moreover, introduction of toxin A and B EIA as an intermediate test between GDH and molecular detection could reduce costs, as it permits to save one third of the molecular tests needed for confirmation.

Laboratory diagnosis of CDI is undergoing changes. A few years ago, most diagnostic laboratories performed toxin A and/or B EIA as the only diagnostic test; in other words, 1 in every 2 CDI cases went undiagnosed and almost 50% of positive results were false-positive (given a theoretical prevalence of 10%).<sup>7</sup> The increased concern generated by the NAP1/027 epidemic hastened recovery of the “silent” GDH antigen and use of multistep GDH-based algorithms that almost double the sensitivity of toxin EIAs with specificity values near 100%. Moreover, the development of commercial molecular methods—albeit with a slight increase in cost—made it possible to reduce the turnaround time of these algorithms to a few hours when they were included as confirmatory tests. The ever increasing number of commercially available molecular tests and decreasing production costs thanks to the development of new technologies will probably lead to a substantial reduction in the final price of molecular tests, thus ensuring in the near future their widespread use by diagnostic laboratories as stand-alone tests for the diagnosis of CDI.

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