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Controversies in *Clostridium difficile* Testing

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Abstract

Recent reports of two nosocomial outbreaks of *Clostridium difficile*-associated disease caused by toxin A-deficient strains emphasize that strains can cause disease. Laboratories using an assay that detects only toxin A as their primary diagnostic test risk misdiagnosis of cases or outbreaks in the institutions they serve. Repeat testing can account for a significant portion of a laboratory’s *C. difficile* testing workload. Published data are available to support laboratory rules for rejection of repeat stool specimens within 7 days of an initial specimen. There are also substantial published data to support laboratory rejection of formed stools sent to the laboratory for *C. difficile* testing.

Introduction

*Clostridium difficile* is associated with 90 to 100% of cases of pseudo-membranous colitis, 60 to 75% of antibiotic-associated colitis, and 11 to 33% of antibiotic-associated cases of diarrhea (1). Asymptomatic carriage is thought to be two- to five-fold more common than the disease itself. The organism produces two exotoxins: toxin A, which is thought to be responsible for most of the gastrointestinal symptoms seen, because it functions as an enterotoxin; and toxin B, which is a potent cytotoxin. A working definition of *C. difficile*-associated disease (CDAD) includes (i) diarrhea, defined as at least six watery stools over 36 h, three unformed stools in 24 h for 2 days, or eight unformed stools over 48 h; (ii) pseudomembranes seen at endoscopy of the lower gastrointestinal tract or detection of toxins or toxigenic organisms in stool; (iii) no other recognized etiology for diarrhea; and (iv) a history of treatment with antimicrobial or antineoplastic agents within the previous 8 weeks or a response to specific therapy for CDAD (2). Many antimicrobial and antineoplastic agents have been demonstrated to induce CDAD, but the most common are the antimicrobial agents clindamycin, ampicillin, amoxicillin, and the cephalosporins (3).

Diagnosis of CDAD is important because the disease can be severe and it is a major nosocomial problem. A variety of methods are currently available for the laboratory diagnosis of CDAD.
including cell culture assays for the presence of cytotoxin, anaerobic culture of stool for the organism followed by testing for the production of toxin (toxicogenic culture), latex agglutination for the detection of C. difficile-associated antigen in stool, and enzyme immunoassays (EIAs) for the detection of toxin A or toxin B, or both. EIA formats include both microdilution and immunochromatographic tests. Rapid laboratory diagnosis of CDAD is highly desirable in the setting of hospital cost containment, which makes an EIA for the detection of toxins in stool specimens a desirable testing option. For this reason, many laboratories now perform EIAs for the detection of toxins in stool specimens. Table 1 shows the results of a recent e-mail survey to which clinical microbiologists from 56 laboratories across the United States responded. These clinical microbiology laboratories ranged in size from a small laboratory serving a single hospital with 139 beds to a large laboratory serving multiple hospitals with a total of 2,400 beds. A clear majority of respondents (71%) perform an EIA as their primary assay for the diagnosis of CDAD.

A multitude of concerns and questions exist about laboratory diagnosis of CDAD. A major concern is which assay to perform as the primary test. Laboratory must first decide which analyte to test for (whether the organism itself through culture, or antigen, or toxin) and which format (cell line, microdilution plate, or immunochromatography) best fits into their workflow and suits the demands of their customers. This article presents data that may influence the decision as to which toxin should be the analyte. I will not discuss the performance characteristics of individual test kits. The decision of which specific test kit to use should be made following a thorough literature search and an in-house comparison study. I will present data to help laboratorians avoid repeat testing and requests to detect C. difficile or its toxins in asymptomatic patients.

The Primary Assay Should Not Be for Toxin A Alone

When the primary assay for the diagnosis of C. difficile disease is the cytoxin assay, toxin B is sought in stool specimens. There is a case report in the literature of a toxin B gene-deficient isolate (4) associated with CDAD. The patient in this report clearly had diarrhea, and C. difficile was isolated from the patient's stool. The PCR primer pairs used by the investigators demonstrated the presence of the toxin A gene, but the toxin B gene-specific primers failed to promote amplification of the portion of the toxin B gene they were designed to recognize. Thus, at least a portion of the toxin B gene was missing from the isolate of C. difficile. Interestingly, the authors of the case report imply that the cytotoxin assay was positive during the patient's diarrheal episode from which the toxin B-deficient C. difficile isolate was obtained. Therefore, it appears the diagnosis of CDAD caused by organisms such as this isolate can be made using the cytotoxin assay. It is also possible that the patient was infected with two different strains of C. difficile, one that was toxin B-deficient and one that was not. At the present time, the detection of toxin B alone should suffice for the diagnosis of CDAD.

Laboratories that rely on an assay for the detection of toxin A alone may want to reconsider their choice of assays to diagnose CDAD. Isolates that lack a portion of the toxin A gene have been known for some time (5,6). These organisms were once thought to be unable to cause disease in humans (7), but evidence that these isolates can cause disease is now beginning to surface. A case of pseudomembranous colitis caused by a toxin A-negative, toxin B-positive strain of C. difficile was recently reported from Seattle, WA (8). Stool specimens from the patient gave a negative result with a toxin A EIA and a positive result with the cytotoxin assay. PCR performed using DNA extracted from the C. difficile strain isolated from the patient's stool demonstrated a truncated (1.7-kb deletion) toxin A gene. The missing portion of the gene coded for the epitope (PCG-4 epitope) that reacts with the monoclonal antibody in the EIA that was used to detect toxin A in stool. There have been two recently described nosocomial outbreaks of CDAD caused by toxin A-negative, toxin B-positive strains of C. difficile (9-11). The first report is from Winnipeg, Manitoba, Canada and describes an outbreak involving 16 patients with antibiotic-associated diarrhea or pseudomembranous colitis (9,10). The routine diagnostic test used by the hospital laboratory for diagnosis of CDAD was an EIA that detected toxin A alone. The index case was a patient for whom there was a high clinical suspicion of CDAD but whose stools were negative for C. difficile toxin on three separate occasions when tested by the enzyme immunoassay for toxin A. The diagnosis of CDAD was made by using a cytotoxin assay. Over the next 2 weeks, three additional patients had similar presentations with stool specimens negative by toxin A EIA and positive by a cytotoxin assay. The remaining cases were identified using both a toxin A EIA, which was negative, and an EIA capable of detecting both toxins A and B, which was positive. Cytotoxigenic C. difficile isolates with identical pulsed-field gel electrophoresis patterns were isolated from seven patients. PCR analysis demonstrated that these seven isolates had a 1.8-kb deletion from the carboxy repetitive oligopeptide region of the toxin A gene, which encodes the epitope recognized by the antibody to toxin A in the EIA used as the diagnostic test for C. difficile.

The second outbreak was reported from Meibergdreef, Amsterdam (11). Toxin A-negative, toxin B-positive infections were recognized by the unique cytotoxic effect these organisms produce in the cytotoxin assay described previously by Kato et al. (7). Discrete clusters of rounded cells were seen rather than cell rounding with disrupted cell-to-cell contact displayed by toxin-A-positive, toxin B-positive strains. This outbreak involved 24 patients whose symptoms ranged from mild to severe; one death occurred. Identical PCR ribotyping and arbitrarily primed PCR patterns were identified for 15 of 16 isolates from the patients involved in the outbreak. Four representative isolates of these toxin A-negative strains had a truncated toxin A gene that was 1.7 kb smaller than expected. These four isolates gave negative results when tested for the production of toxin A by an EIA that detected toxin A alone. The reports described above demonstrate that toxin A-negative, toxin B-positive organisms can cause disease, and the presence of these organisms in stool specimens cannot be detected by
using an EIA that detects toxin A alone. How common are these isolates and how often do they cause disease? These questions are still unanswered. In Japan, Kato et al. (7) reported isolation of these organisms from 6.7% of symptomatic children, 12.5% of asymptomatic children, and 12.5% of asymptomatic adults. Burday et al. (12) report that the incidence of stool specimens that were toxin A negative by EIA but positive by a toxin A+B EIA in two hospitals in New Jersey was 17 and 34%. The initial reports from Kato et al. and the case report and two outbreaks discussed earlier indicate these organisms are found worldwide. These reports make it clear that testing for toxin A alone may lead to undiagnosed cases or outbreaks of CDAD. Therefore, testing for toxin A alone is not without risk, and laboratories testing for toxin A alone should consider switching to the cytotoxin assay or an assay that detects both toxins A and B.

The Biosite Triage C. difficile panel (Biosite Diagnostics, San Diego, CA) simultaneously detects toxin A and the C. difficile common antigen, glutamate dehydrogenase. Laboratories using this assay should expect to frequently see test results with only the common antigen positive because the panel is more sensitive for the common antigen than it is for toxin A (13,14). Also, organisms that produce neither toxin A nor B are common in the environment and in stool specimens. Use of the Biosite Triage test requires that the laboratory have a second assay to interpret toxin A-negative, common-antigen-positive results. The confirmatory assay should be the cytotoxin assay or an EIA that detects both toxins A and B.

**Reject Those Repeat Specimens**

Repeat testing ordered by physicians can account for a significant number of the test requests for C. difficile toxin assays received by a laboratory. Renshaw et al. (15) reported that repeated testing within 7 days of an initial request accounted for 36% of their C. difficile cytotoxin assay requests. It is very easy for laboratories to reject repeat test requests for patients whose previous C. difficile toxin test was positive, regardless of the assay the laboratory uses. The Society for Healthcare Epidemiology of America (SHEA) position paper (2), which summarizes and analyzes the results of many published studies, says that "test-of-cure cultures or toxin assays following treatment are not recommended, as they are imperfect predictors of subsequent relapse." SHEA also believes that patients should not be considered therapeutic failures until they have received at least 6 days of treatment. Both of these opinions can be cited by the laboratory to reject repeat test requests within 7 days of a previous positive result.

The cytotoxin assay is the only assay for which there are published data concerning the rejection of repeat requests following an initial negative result. The study published by Renshaw et al. (15) is aptly named "The lack of value of repeated Clostridium difficile cytotoxicity assays." Renshaw et al. described 947 episodes in which two or more cytotoxicity assays were repeated within 7 days of an initial request. This repeat testing provided useful information (a change from negative to positive) in only 0.8% of cases. The authors concluded that C. difficile cytotoxicity assays should not be repeated within a 7-day period.

A retrospective review revealed that 20% of our requests for C. difficile cytotoxin assays were repeat tests within 7 days of a previous request, and useful information was obtained in only 0.5% of these repeat tests. Despite a move from the cytotoxin assay to a toxin A + B EIA that performed well in an in-house comparison of cytotoxin and culture, we decided to institute the rule of no repeat testing within 7 days in our institution. Physicians who want repeat testing must call the laboratory to prevent rejection of their request. Only our intensive care unit asked not to be included. After implementing the 7-day rule, we saw a 35% decrease in the number of tests per patient, from 3.1 to 2.0. From May 2000 to June 2001, when we performed toxin A + B EIA testing only, we received 74 sets of stool specimens with two or more requests for C. difficile toxin testing, and only 2 (2.7%) of these sets provided clinically useful results. I feel our rejection criteria have been successful and encourage others to institute such a policy.

**Don’t Test Stools from Asymptomatic Patients**

Some laboratories may be asked to search for C. difficile or its toxins in stool specimens (formed stools) from asymptomatic patients in an effort to identify, isolate, or even treat carriers to try to control the spread of nosocomial CDAD. Caregivers and infection control practitioners may have been influenced by a recent article published in the American Journal of Infection Control, which describes the policy of identifying readmitted patients who were positive for C. difficile within the past 6 months (16). These patients are placed in isolation and tested for the presence of C. difficile toxin. Patients whose stool specimens are toxin negative are removed from isolation; toxin-positive patients remain isolated and are treated for CDAD (generally with 7 to 10 days of oral metronidazole).

Although the authors attribute a decline in the number of new patients with CDAD to the institution of this policy in 1995, they fail to discuss the significance or role of changes in housekeeping, hand washing, glove wearing, education, etc., that occurred in response to an outbreak of CDAD in their institution in 1994 (17). The authors also endorse C. difficile toxin testing as a test of cure.

Published data support the contention that stool specimens from asymptomatic patients should not be tested for the presence of toxin-producing C. difficile in an effort to prevent them from developing disease as a result of their own colonized state. Patients who are asymptomatically colonized on admission actually have a very low risk of subsequent development of CDAD (18). Primary symptomless colonization may provide patients with protection from developing CDAD (19). This protection appears to be provided by both toxigenic and nontoxigenic strains.

Published data also support not testing stool specimens from asymptomatic patients for the presence of toxigenic C. difficile in an effort to prevent nosocomial transmission of the organism. Not every patient in the hospital who acquires C. difficile is at risk for the development of CDAD. Clinical symptoms develop in only about 33% of hospitalized patients who become colonized (3). The SHEA position paper reminds us that transmission of infection to other patients is associated with ongoing diarrhea and not with the mere
presence of toxin in the stool (2). Testing patients to identify and treat carriers in an effort to eradicate the carrier state is also not justified because neither vancomycin nor metronidazole, nor any other antimicrobial regimen, has been demonstrated to be reliably effective in this effort (20, 21).

Summary

We now know that toxin A-negative, toxin B-positive strains of *Clostridium difficile* can cause CDAD and that these organisms are found worldwide. The presence of these organisms cannot be detected by using an assay that detects toxin A alone. Laboratories that use an assay that detects toxin A alone risk misdiagnosis of individual cases or even outbreaks of CDAD. Laboratories can significantly reduce their *Clostridium difficile* testing workload by instituting a policy of no repeat testing within 7 days of a previous test. However, flexibility is needed in enforcing this policy to allow repeat testing after consultation with the patient’s physician in cases of high suspicion when the initial test result is negative. Testing asymptomatic individuals provides no useful clinical data and should not be used to identify carriers for purposes of infection control or treatment to eliminate the carrier state.

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### Table 1. *Clostridium difficile* testing survey results from 56 clinical microbiology laboratories

| Primary Assay                  | No. of laboratories performing assay | Percent of laboratories performing assay |
|-------------------------------|-------------------------------------|-----------------------------------------|
| Microdilution EIA             | 40                                   | 71                                      |
| Toxin A + B                   | 24                                   | 43                                      |
| Toxin A                       | 16                                   | 29                                      |
| Cytotoxin                     | 11                                   | 20                                      |
| Membrane EIA                  | 5                                    | 9                                       |
| Toxin A + antigen             | 4                                    | 7                                       |
| Toxin A                       | 1                                    | 2                                       |
| Toxigenic culture             | 0                                    | 0                                       |

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