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Effective Detection of Toxigenic *Clostridium difficile* by a Two-Step Algorithm Including Tests for Antigen and Cytotoxin

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We evaluated a two-step algorithm for detecting toxigenic *Clostridium difficile*: an enzyme immunoassay for glutamate dehydrogenase antigen (Ag-EIA) and then, for antigen-positive specimens, a concurrent cell culture cytotoxicity neutralization assay (CCNA). Antigen-negative results were ≥99% predictive of CCNA negativity. Because the Ag-EIA reduced cell culture workload by ≈75 to 80% and two-step testing was complete in ≤3 days, we decided that this algorithm would be effective. Over 6 months, our laboratories' expenses were US$143,000 less than if CCNA alone had been performed on all 5,887 specimens.

Clostridium difficile-associated diarrhea is an important illness among patients who are extensively treated with antibacterial or other chemotherapeutic agents (4, 7, 19). While definitive evidence of toxigenic *C. difficile* comes from microbiologic testing, laboratories are challenged to provide accurate results rapidly and cost-effectively (23). Cell culture assays for cytotoxin (toxin B) are considered the gold standard but require up to 4 days for results, expensive cells and media, and labor-intensive expertise (4, 17, 19).

Consequently, many laboratories use immunoassays for *C. difficile* toxins, or "common" glutamate dehydrogenase antigen (2, 6, 13, 16, 19, 21, 23, 24). Toxin enzyme immunoassays (EIAs) are frequently used as stand-alone assays but clinical sensitivity may be suboptimal, particularly if only toxin A is detected (4, 6, 8, 9, 12, 19). Current antigen EIAs (Ag-EIAs) accurately detect an essential and constitutively synthesized enzyme (23), thereby rapidly identifying *C. difficile* while overcoming the low sensitivity of toxin EIAs and suboptimal performance of older, latex-agglutination antigen assays. Because Ag-EIAs detect nontoxigenic as well as toxigenic *C. difficile*, however, they must be used in combination with a toxin-detecting assay to provide specific laboratory evidence of *C. difficile*-associated diarrhea (2, 3, 10, 11, 16, 19, 21–24).

Both of our institutions' *C. difficile*-testing laboratories had adopted a stand-alone EIA approach for detecting toxins A and B, using *C. DIFFICILE TOX A/B II* (ToxAB-EIA; TechLab, Blacksburg, Va.; distributed by Wampole Laboratories, Princeton, N.J.). These laboratories are in acute-care hospitals: one in the 900-bed Johns Hopkins Hospital (JHH), which also serves the 190-bed acute-care Howard County General Hospital in Columbia, Md.; the other is at the 350-bed Johns Hopkins Bayview Medical Center (JHBMC) and serves the 220-bed Johns Hopkins Care Center, a colocated facility for subacute and long-term care. During late 2003, it became apparent that the sensitivity of the ToxAB-EIA was unacceptably low at JHH (see below for analogous JHBMC data from 2004). After determining that the performance of similar assays was inadequate (unpublished data; e.g., 71% sensitivity, 73% specificity, and 25% negative predictive value for Premier *C. difficile* Toxin A+B [Meridian Diagnostics, Cincinnati, Ohio] versus cytotoxin testing for 63 specimens), we decided to develop an alternative approach.

Preimplementation evaluation. Our ultimate goals were high sensitivity and adequate turn-around times while reducing workload and cost that would have resulted from cell culture testing of all specimens. We therefore evaluated a testing process of two steps: an Ag-EIA (*C. DIFF CHEK-60*; TechLab/Wampole) and, for antigen-positive specimens, a concurrent cytotoxicity neutralization assay (CCNA) in cell culture. This evaluation was conducted with 366 fecal specimens that had been submitted to our two clinical laboratories for evidence of toxigenic *C. difficile*. For CCNA, a previously described method (5, 17) was modified to yield final results more rapidly: human foreskin fibroblasts were simultaneously inoculated with two aliquots of specimen, one of which had been incubated with anti-toxin B (TechLab), and then observed for cytopathic effect at 24 and 48 h. We concurrently determined ToxAB-EIA sensitivity at our second laboratory. Data were primarily analyzed according to the laboratory to which specimens were submitted because each serves somewhat different populations and because the study designs were not identical.

Among the total of 366 specimens, 266 were submitted to the JHH Virology Laboratory during January 2004. For this study, each specimen was tested by Ag-EIA and CCNA (Table 1). The Ag-EIA was very likely to identify potentially CCNA-positive specimens: 96% sensitivity and 99.5% predictive value of an antigen-negative result. While the predictive value of an antigen-positive result was 49%, Ag-EIA screening would have led to an 82.3% decrease in CCNA testing; i.e., only the 47 antigen-positive specimens would have been CCNA tested.

The other 100 specimens were submitted during June and July 2004 to the JHBMC Microbiology Laboratory. Each specimen was EIA tested for antigen and toxins A and B (ToxAB) (Table 2). CCNA was performed on all 27 antigen-positive and 10 randomly selected antigen/ToxAB-negative specimens. Our

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TABLE 1. Results of testing 266 Johns Hopkins Hospital specimens for *C. difficile* cytotoxin and glutamate dehydrogenase antigen

Ag-EIA result	Cytotoxin result[a] (no. of specimens)		Frequency of positive results (%)		% Sensitivity (95% CI)[d]	% Specificity (95% CI)	PVP[b] (95% CI)	PVN[c] (95% CI)
	Positive	Negative	Cytotoxin	Ag-EIA				
Positive	23	24	9[e]	18	96 (≥79)	90.1 (85.6–93.5)	49 (34–64)	99.5 (≥97.5)
Negative	1	218						

[a] All 266 specimens were tested for cytotoxin by concurrent CCNA and for glutamate dehydrogenase antigen by Ag-EIA.
[b] PVP, predictive value of a positive Ag-EIA result. Values are percentages.
[c] PVN, predictive value of a negative Ag-EIA result. Values are percentages.
[d] 95% CI, 95% confidence interval.
[e] This frequency was used as an approximation of toxigenic *C. difficile* prevalence.

decision to CCNA test only 10 antigen/ToxAB-negative specimens was based on the 99.5% predictive value of an antigen-negative result with JHH specimens at ≈9% prevalence (Table 1) and on previous reports of 98.7% negative predictive value at 13.8% prevalence (24) and 100% negative predictive value at 11.6% prevalence (21) with this *C. DIFF CHEK-60* assay. Likewise, others have reported very high negative predictive value with a similar Ag-EIA, particularly when coupled with negative toxin EIA results (2, 3, 10, 11, 16, 22).

EIA performance was calculated with reference to detectable cytotoxin: a specimen was considered to be cytotoxin positive if it was CCNA positive and cytotoxin negative if it yielded negative results by CCNA or both EIAs. CCNA-positive results were obtained from all six antigen/ToxAB-positive and 10 of the 21 antigen-positive/ToxAB-negative specimens (Table 2). All 10 antigen/ToxAB-negative specimens were CCNA negative. CCNA and Ag-EIA findings were otherwise similar to those for JHH specimens, including a 73% CCNA decrease via Ag-EIA screening.

Overall, 303 (83%) of 366 specimens were assayed by CCNA; all were tested by Ag-EIA. The antigen-positive frequency was 20% (74 of 366). Using our criterion for cytotoxin negativity, the cytotoxin-positive frequency was 11% (40 of 366); Ag-EIA sensitivity, specificity, positive predictive value, and negative predictive value were 98, 89.3, 53, and 99.7%, respectively. Ag-EIA screening would have reduced CCNA workload by 79.8%.

Postimplementation evaluation. We assessed first-step effectiveness by using two indirect measures, distributions of optical density (OD) values and episodes with potential false-negative results, to determine if antigen-negative results were highly predictive of cytotoxin negativity.

First, we analyzed ODs from 25 Ag-EIA runs. Among 292 antigen-negative results, none were within 10% of the cutoff OD of 0.120 at 450 nm, and 3 (1%) were within 20%; their ODs were 0.101, 0.103, and 0.107. One (1%) of 94 antigen-positive ODs, at 0.130, was within 10 or 20% of the cutoff. The distribution of antigen-negative ODs was near normal with a skew toward higher ODs (range, 0.031 to 0.107; 95th-percentile range, 0.033 to 0.059; mean, 0.046; median, 0.045; mode, 0.042); it was more broad than but otherwise similar to the distribution of 25 negative-control ODs (range, 0.033 to 0.059; mean, 0.044; median and mode, 0.045). ODs varied more among antigen-positive specimens (95th-percentile range, 0.198 to >3; 68% were >3, the maximum measurable OD).

Second, we defined an episode as a 7-day period during which at least two specimens were collected from a patient, and we defined potentially false-negative as initially antigen-negative and subsequently cytotoxin-positive results during an episode. Among 351 JHBMC episodes between December 2004 and May 2005, seven had potential false-negative results, so there was a maximum yield of 2% additional cytotoxin-positive results from testing more than one specimen. For six episodes, potential false-negative ODs ranged from 0.038 to 0.057. The seventh episode's potential false-negative ODs were 0.050 and 0.101, whereas the antigen-positive OD was 0.198. For this episode and one other, all three specimens were collected on the same day; the other five episodes had 3- to 5-day intervals between potential false-negative and cytotoxin-positive specimens.

TABLE 2. Results of testing 100 Johns Hopkins Bayview specimens for *C. difficile* cytotoxin, glutamate dehydrogenase antigen, and toxin A and toxin B antigens

EIA	EIA result	Cytotoxin result[a] (no. of specimens)		Frequency of positive results (%)		% Sensitivity (95% CI)	% Specificity (95% CI)	PVP[c] (95% CI)	PVN[c] (95% CI)
		Positive	Negative	Cytotoxin	EIA				
Antigen	Positive	16	11	16[b]	27	100 (≥79)	87 (78–93)	59 (39–78)	100 (≥95)
	Negative	0	73						
ToxAB	Positive	6	0		6	38 (15–65)	100 (≥96)	100 (≥54)	89 (81–95)
	Negative	10	84						

[a] All 100 specimens were tested by enzyme immunoassays for glutamate dehydrogenase antigen (Ag-EIA) and for toxins A and B (ToxAB-EIA); 37 were tested for cytotoxin by CCNA, including all 27 Ag-positive and 10 randomly selected Ag/ToxAB-negative specimens. See text for explanation and results. Also see Table 1, footnotes *b*, *c*, and *d*.
[b] This frequency was used as an approximation of toxigenic *C. difficile* prevalence.
[c] Values are percentages.

TABLE 3. Two-step *C. difficile* testing of 5,887 specimens at Johns Hopkins Bayview Medical Center and Johns Hopkins Hospital laboratories, December 2004 through May 2005[a]

Laboratory	No. of specimens	Positive-result frequencies (%)		PVP[b]	Reductions[c]		
		Ag-EIA	Cytotoxin		CCNA[d]	Cost/month (US$)	Cost/specimen (US$)
JHBMC	1,579 (226–310)	24.7 (23–28)	15.3 (11–19)	61.8 (47–72)	75.3 (72–77)	5,700	22
JHH	4,308 (681–806)	16.2 (13–20)	≈8 (7–9)	≈45 (41–50)	83.8 (80–87)	18,100	25

[a] First 6 months after implementation at both laboratories. All specimens were tested by enzyme immunoassay for glutamate dehydrogenase antigen (Ag-EIA). The range of values for each of the 6 months is shown in parentheses.
[b] PVP, predictive value of Ag-positive result. Values are percentages. Estimated JHH values for cytotoxin and PVP were calculated from preimplementation sensitivity and specificity (Table 1).
[c] CCNA reduction equals 100 − %Ag-positive. Cost/month reduction equals CCNA savings less Ag-EIA costs divided by six (analyzed number of months) and rounded to the nearest US$100; CCNA savings equal number of specimens not CCNA tested multiplied by US$39.80 (US$26.80 for 35 min of technologist effort plus US$13 for cell culture supplies); Ag-EIA costs equal number of specimens multiplied by US$8.30 per JHBMC specimen (US$4.60 for 6 min of technologist effort plus US$3.70 for Ag-EIA materials) and US$8.10 per JHH specimen (US$3.50 for Ag-EIA materials). Cost/specimen reduction equals total cost reduction divided by total number of specimens.
[d] Values are percentages.

We also calculated laboratory savings for the first 6 months that two-step *C. difficile* testing was available at both laboratories (Table 3). Because pertinent data were more readily obtained from the JHBMC information system, we analyzed Ag-EIA and CCNA results for all JHBMC specimens. For JHH specimens, we had monthly volumes of tested and antigen-positive specimens; from these volumes, we estimated cytotoxin-positive frequencies and positive predictive value by using pre-implementation values for Ag-EIA sensitivity and specificity from Table 1.

Estimated JHH cytotoxin positivity frequency was similar to that during late 2003 (5); both laboratories' antigen-positive frequencies were similar to those obtained before implementation, as were JHBMC's cytotoxin-positive frequencies and positive predictive value (indicating that preimplementation specimen collections were representative of the populations

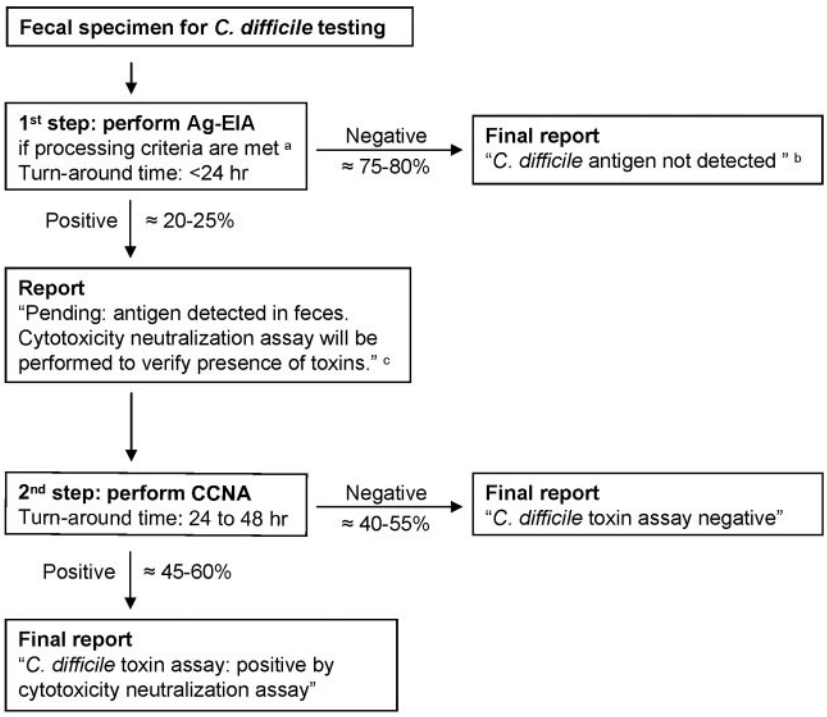

FIG. 1. Flow chart for two-step testing algorithm to detect toxigenic *C. difficile*. Ag-EIA, enzyme immunoassay for glutamate dehydrogenase antigen; CCNA, concurrent cell culture cytotoxicity neutralization assay. Superscript letters: a, testing for a patient is automatically rejected if (i) two specimens have already been processed during the preceding 7 days or (ii) the specimen was collected within 30 days of a CCNA-positive result; b, if clinical suspicion of *C. difficile*-associated diarrhea is still high, laboratories suggest a new order for repeat testing with CCNA; c, quote is from the JHH laboratory information system; at JHBMC, the information system reports state "antigen detected: may not correlate with disease, see toxin results."

that each laboratory served). Between December 2004 and May 2005, Ag-EIA screening reduced our laboratories' CCNA workload by 81.5%. Total expenditures were 61.0%, or US$143,000, less than the cost of performing CCNA alone on every specimen. JHBMC per-specimen savings were smaller, primarily because a higher proportion of its specimens were CCNA tested.

Clinical laboratories can be overwhelmed with requisitions for *C. difficile* testing, regardless of the methods that are used, because medical institutions like ours frequently have very ill patients with diarrhea. The results herein demonstrate a practical approach to sensitive and efficient detection of toxigenic *C. difficile* by using a combination of Ag-EIA and CCNA (Fig. 1). This two-step testing process is now used for all Johns Hopkins patients because it met our goals for sensitivity, turn-around time, workload, and cost.

Two-step *C. difficile* testing replaced the use of a ToxAB-EIA at our institutions. ToxAB-EIA sensitivity was 38% (Table 2) and much lower than the range of 66 to 94% in earlier reports (1, 14, 16, 18, 21) and Food and Drug Administration-cleared package inserts (TechLab/Wampole). This low sensitivity was consistent with our physicians' suspicions of higher *C. difficile*-associated diarrhea incidence than revealed by laboratory testing, which had led to a practice of multiple requisitions per patient. While we did not attempt to identify reasons for our low-sensitivity ToxAB-EIA data, another study determined that false-negative EIA results for toxin A correlated with low titers of toxin B in cell culture (10). We concluded that this ToxAB-EIA was not appropriate for our patients.

In contrast, our 96% frequency of antigen-positive results among cytotoxin-positive specimens (Table 1) was similar to previously reported sensitivities, 92.7 and 100%, for the same Ag-EIA (21, 24). While our postimplementation evaluation of 351 multispecimen episodes identified seven (2%) with potential false-negative results, this analysis had a conservative bias: intervals of 4 to 5 days between potential false-negative and cytotoxin-positive specimens may have indicated *C. difficile*-associated diarrhea only during the late part of three such episodes. Because (i) our group and two others have obtained high Ag-EIA sensitivity and negative predictive value, (ii) OD distributions indicated wide separation between the vast majority of antigen-negative and antigen-positive results, (iii) few specimens yielded close-to-cutoff ODs, and (iv) the proportion of episodes with potential false-negative results was small, we concluded that false antigen-negative results were very infrequent and that the Ag-EIA was suitable as a rapid first-step assay for detecting toxigenic *C. difficile*.

Our physicians therefore obtain useful information within 1 day of specimen receipt (Fig. 1), via reporting of Ag-EIA results: "antigen not detected" is highly predictive of cytotoxin negativity, whereas "antigen detected" is reported with "pending" while CCNA is performed. We incubate CCNA for as long as 48 h but 75 to 80% of cytotoxin-positive specimens are identified after 24 h (17). The testing process is therefore complete within 3 days but most antigen/CCNA-positive specimens are identified within 2 days, at least as rapidly as by the reference assay in which the neutralization component is performed after cytopathic effect is observed. Moreover, this timeline is similar to that observed when multiple samples were submitted for toxin-EIA testing to achieve 90% accuracy (15).

Our cytotoxin-positive frequencies among antigen-positive specimens (positive predictive value in Tables 1 to 3) were similar to those reported previously (2, 3, 10, 11, 16, 21–24). Although such low frequencies might appear to preclude first-step use of the Ag-EIA, the workload-diminishing effect of the two-step process renders it cost-effective to test all antigen-positive specimens concurrently for cytotoxicity and neutralization. Antigen-negative results would have eliminated nearly 80% of our preimplementation CCNA testing; this prediction was verified after implementation (Table 3).

Others have obtained antigen results like ours by studying the same *C. DIFF CHEK-60* (21, 24) or the Triage *C. difficile* panel (Biosite Diagnostics, San Diego, Calif.) that separately detects antigen and toxin A via membrane-EIA (2, 3, 10, 11, 16, 22). Several of these investigators recognized the potential value of Ag-EIAs in screening for toxigenic *C. difficile*, especially as an alternative to isolating *C. difficile* in culture. In particular, Landry et al. (10) proposed a two-step test that consisted of the Triage panel and CCNA for antigen-positive/toxin A-negative specimens. This approach had the advantage of rapidly identifying certain toxigenic (toxin A-positive) strains, with a ≈75% reduction in cell culture workload (similar to ours because Triage toxin A sensitivity was 33%). Snell et al. (21) recently recommended a three-step approach, consisting of Ag-EIA, toxin-EIA, and CCNA. The relative simplicity of our algorithm eliminates the possibility of false-positive toxin-EIA results, while yielding sensitivity, turn-around time, and cost-effectiveness similar to those assays discussed above.

Our cost-effectiveness has been further increased by laboratory policy, implemented along with two-step testing, that limits *C. difficile* testing for each patient. Testing is not routinely performed on more than two specimens during a 7-day interval or on any specimen for 30 days after a cytotoxin-positive result (Fig. 1). This policy is based on studies in which third-specimen testing identified <1% additional *C. difficile*-associated diarrhea and on the likelihood of detectable cytotoxin even after successful treatment (4, 5, 19, 20). Providers may, however, request testing outside of these limitations, thereby addressing the unusual possibility of a false antigen-negative result (5, 15).

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