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Impact of Clinical Symptoms on Interpretation of Diagnostic Assays for *Clostridium difficile* Infections▽

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Asymptomatic *Clostridium difficile* colonization is common in hospitalized patients. Existing *C. difficile* assay comparisons lack data on severity of diarrhea or patient outcomes, limiting the ability to interpret their results in regard to the diagnosis of *C. difficile* infection (CDI). The objective of this study was to measure how including patient presentation with the *C. difficile* assay result impacted assay performance to diagnose CDI. Stool specimens from 150 patients that met inclusion and exclusion criteria were selected. Nine methods to detect *C. difficile* in stool were evaluated. All patients were interviewed prospectively to assess diarrhea severity. We then assessed how different reference standards, with and without the inclusion of patient presentation, impact the sensitivity, specificity, and positive and negative predictive values of the assays to diagnose CDI. There were minimal changes in sensitivity; however, specificity was significantly lower for the assays Tox A/B II, C. diff Chek-60, BD GeneOhm Cdiff, Xpert C. difficile, and Illumigene C. difficile and for toxigenic culture (*P* was <0.01 for all except Tox A/B II from fresh stool, for which the *P* value was 0.016) when the reference standard was recovery of toxigenic *C. difficile* from stool plus the presence of clinically significant diarrhea compared to when the reference standard was having at least four assays positive while ignoring diarrhea severity. There were 15 patients whose assay result was reported as negative but subsequently found to be positive by at least four assays in the comparison. None suffered from any CDI-related adverse events. In conclusion, clinical presentation is important when interpreting *C. difficile* diagnostic assays.

Clostridium difficile infection (CDI) is the leading cause of infectious nosocomial diarrhea in North America and Europe (3). CDI incidence and severity have been increasing for the last decade. These changes in CDI epidemiology have brought renewed focus on methods to detect *C. difficile* and/or its toxins in stool.

Because of the lower sensitivity to detect the presence of toxigenic *C. difficile* in stool versus other methods, the Society for Healthcare Epidemiology of America and Infectious Diseases Society of America CDI guidelines state that toxin enzyme immunoassays (EIAs) are a suboptimal approach for the diagnosis of CDI (3). An alternative was not recommended, leaving laboratory personnel to interpret the *C. difficile* diagnostic literature when choosing a substitute. Although the performance of *C. difficile* assays relative to each other can be made, contemporary studies suffer from the use of different reference standards and inconsistent reporting on the source and consistency of specimens (23). More importantly, the current CDI diagnostic literature does not include prospective patient interviews to assess clinical symptoms, medication exposures, and/or patient outcomes. CDI is a clinical diagnosis supported by laboratory or endoscopic evidence, not vice versa. The lack of clinical data is problematic, because asymptomatic *C. difficile* colonization and diarrhea are common in populations exposed to health care facilities (3, 15, 16). It is not possible to determine the true meaning of a positive *C. difficile* assay and how it relates to the diagnosis of CDI in the absence of patient symptoms and outcomes (14).

In April 2009, the *C. difficile* toxin EIA used by our institution became unavailable due to manufacturing difficulties (ProSpecT *Clostridium difficile* Toxin A/B; Remel, Lenexa, KS). A significant drop in CDI incidence was noted when a replacement toxin EIA was implemented (*C. difficile* Tox A/B II; TechLab, Blacksburg, VA). An epidemiological study failed to identify increased CDI related morbidity or mortality, suggesting the difference was due to improved specificity of the new assay (7). To confirm this, and because of the limitations of published studies, a formal assay comparison that included prospective patient interviews and an assessment of outcomes was conducted. These data were used to test the hypothesis that the apparent specificity of any given *C. difficile* assay for the diagnosis of CDI will decrease when clinical presentation of the patient is included in the reference standard.

(These data were presented in part at the 21st Annual Meeting of the Society for Healthcare Epidemiology of America, 1 to 4 April 2011, Dallas, TX.)

MATERIALS AND METHODS

Patient population and stool specimen collection. The study was conducted at Barnes-Jewish Hospital (BJH), in St. Louis, MO. The BJH microbiology laboratory only tests unformed stools for *C. difficile*, per published guidelines (3). *C. difficile* testing requires an order from a physician for diagnostic purposes. Unformed stool, defined as stool that conforms to its container, submitted from inpatients for *C. difficile* testing was eligible to be included in the comparison if it was the first specimen sent during that admission and if a minimum of 10 ml of stool was submitted. The specimen was tested and results reported per standard practice with the assay used by the clinical microbiology laboratory during

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the study period (*C. difficile* Tox A/B II). The remaining specimen was divided into 10 1-ml aliquots and frozen at −80°C. Patients were interviewed within 24 h of stool collection. Specimens were excluded if the patient was unable to communicate. The first 100 consecutive specimens that met inclusion and exclusion criteria were included in the study. To better evaluate specificity, an additional 50 specimens that were positive using ProSpecT *Clostridium difficile* Toxin A/B, the assay most frequently positive with the first 100 specimens, were collected. The first 100 specimens were collected between 11 January 2010 and 5 February 2010, and the last 50 specimens were collected between 19 March 2010 and 30 June 2010.

Patients were interviewed by one of two physicians with infectious diseases training (E.R.D. or Z.H.). The first 10 interviews were conducted with both physicians present to standardize the interview process and data collection. The interviewers were blinded to the assay result reported to the treating physician. Demographic data, select comorbidities (inflammatory bowel disease and intra-abdominal surgery in the prior 7 days), and receipt of a laxative in the 48 h prior to stool specimen collection were recorded. The patients were questioned on the consistency and number of bowel movements in the 24 h prior to stool collection and on what was typical for them. Patients selected stool consistency from the Bristol Stool Chart. Patients rated abdominal pain or cramping on a scale from 1 (no pain/cramping) to 10 (most severe pain/cramping imaginable). An abdominal exam was performed, and the peak temperature and white blood cell count within 24 h of stool collection were recorded. The outcome at 60 days (death or recurrent CDI) and empirical CDI treatment for patients whose assay result was reported as negative but found to be positive in the assay comparison were assessed.

Testing of stool specimens. The assays compared included two toxin EIAs (*C. difficile* Tox A/B II and ProSpecT *Clostridium difficile* Toxin A/B), one glutamate dehydrogenase (GDH) EIA (C. diff Chek-60 [TechLab]), and three nucleic acid amplification tests (BD GeneOhm Cdiff [Becton Dickinson, Franklin Lakes, NJ], Xpert *C. difficile* [Cepheid, Sunnyvale, CA], and Illumigene *C. difficile* [Meridian Bioscience, Inc., Cincinnati, OH]). All assays were performed according to the manufacturers' instructions (the *C. difficile* Tox A/B II and C. diff Chek-60 package inserts state that activity may be lost if the specimen is frozen, and the Xpert *C. difficile* does not state whether specimens can be frozen prior to testing). Although a positive result from the ProSpecT *Clostridium difficile* Toxin A/B from fresh stool was part of the inclusion criteria for the final 50 specimens, the results of this assay for the fresh specimen were not included in the comparison because the assay was not performed on the first 100 specimens prior to freezing. If the initial result of any assay was indeterminate, instructions provided by the manufacturer to resolve the results of the assay were followed. If repeat results remained indeterminate, the result recorded for the assay comparison was "negative." The person performing the assays was blinded to the results of toxigenic culture and to the clinical data.

The cytotoxicity cell assay was conducted for all stool specimens. Stool samples were diluted 1:1 in phosphate-buffered saline to a total volume of 1 ml and centrifuged for 10 min at 14,000 × *g*. The supernatant was filtered using a sterile 0.45 μM low-protein-binding syringe filter. The cytotoxicity assay was performed according to manufacturer's directions (*Clostridium difficile* toxin/antitoxin kit; TechLab). HT-29 adenocarcinoma intestinal cells (HTB 38; American Type Culture Collection) grown to confluence in 96-well microtiter plates were used. Samples were tested in duplicate. Cytoxicity was assessed after 24 and 48 h of incubation in a 37°C CO_2 incubator. Samples exhibiting typical cell rounding that was neutralized with antitoxin were recorded as positive.

Toxigenic culture was performed for all specimens. Stool was incubated with an equal amount of 95% ethanol (Sigma-Aldrich, St. Louis, MO) at room temperature for 45 min. The specimen was centrifuged at 2,500 × *g* for 10 min, and the supernatant was discarded. The pellet was inoculated onto prereduced cycloserine, cefoxitin, and fructose agar with lysozyme (Anaerobe Systems, Morgan Hill, CA). Plates were inspected for growth after 48 h in an anaerobic incubator at 35°C. Presumptive *C. difficile* isolates were identified, by characteristic colony morphology, smell, and Gram stain, that were also Pro Disk (Remel) positive and spot indole (Remel) negative (6). All isolates were confirmed to be *C. difficile* positive with RapID ANA II (Remel). Any specimen found to be positive by at least two nonculture methods but not by *C. difficile* culture was inoculated into prereduced cycloserine, cefoxitin, and mannitol broth with taurocholate and lysozyme (Anaerobe Systems) (22). If growth was identified within a week of inoculation, the specimen was subcultured onto prereduced blood agar (Remel). *C. difficile* isolates were identified as described above. All *C. difficile* isolates were grown in chopped meat broth (Anaerobe Systems) for 48 h. Culture was tested for cytotoxicity as described above. The presence of the *tcdB* gene was assessed by PCR using the primers described by Peterson et al. in nontoxigenic *C. difficile* strains recovered from stool for which there was at least one positive nucleic acid amplification assay result (13).

TABLE 1. Reference standards for assay comparisons[a]

Reference standard	Clinically significant diarrhea	Toxigenic *C. difficile* isolated	At least 4 assays positive	Interpretation for assay comparison
A	Yes	Yes	NA	Positive
	No	Yes	NA	Negative
	Yes	No	NA	Negative
	No	No	NA	Negative
B	Yes	Yes	NA	Positive
	No	Yes	NA	Positive
	Yes	No	NA	Negative
	No	No	NA	Negative
C	Yes	NA	Yes	Positive
	No	NA	Yes	Negative
	Yes	NA	No	Negative
	No	NA	No	Negative
D	Yes	NA	Yes	Positive
	No	NA	Yes	Positive
	Yes	NA	No	Negative
	No	NA	No	Negative

[a] The presence of clinically significant diarrhea was ignored when interpreting the reference standard and the assays being compared for standards B and D. NA, not applicable.

Statistical analysis. The *a priori* primary reference standard for the assay evaluation was a patient with clinically significant diarrhea and toxigenic *C. difficile* recovered from stool (standard A) (Table 1) (3). Three alternate reference standards were also evaluated to determine how different reference standards impact assay comparisons. These included (i) having the presence of toxigenic *C. difficile* while ignoring clinical presentation (standard B), (ii) having clinically significant diarrhea and at least four assays positive (standard C), and (iii) having at least four assays positive while ignoring clinical presentation (standard D). Only results from the frozen specimens were used for the "at least four assays positive" criteria. Clinically significant diarrhea was defined as at least three diarrheal bowel movements (type 6 or 7 stool on the Bristol Stool Chart) in the 24 h preceding stool specimen collection or diarrhea plus patient-reported abdominal pain or cramping (14). There were four *C. difficile* isolates that were nontoxigenic but positive for the *tcdB* gene. These stool specimens were classified as negative for toxigenic *C. difficile*.

Sensitivity and specificity and their 95% confidence intervals were calculated for each assay against each reference standard. The prevalence for positive and negative predictive value calculations was based on the results of the respective reference standard on the first 100 specimens alone. The impact of a two-step algorithm, i.e., first screening specimens with a GDH EIA (C. diff Chek-60), was also assessed. The algorithm was positive only if both the GDH and the second assay were positive. The Wilcoxon rank sum test was used to compare continuous and ordinal data. Chi-square and Fisher's exact tests were used for categorical data. Outcomes of patients whose clinical assay was reported as negative but was positive by any of the reference standards were described. PASW v17.0 (IBM SPSS, Inc., Somers, NY) and EpiInfo v6 (CDC, Atlanta, GA) were used for statistical analysis.

RESULTS

The median age of the 150 patients who met inclusion and exclusion criteria was 60 years (range, 21 to 96 years) (Table 2). The race distribution was similar to that typical for BJH (4). The median peak temperature within 24 h of stool collection was 37.1°C (range, 35.6°C to 39.7°C), and 27 patients (18%) had a fever of ≥38.3°C. Patients had significantly more bowel movements per day compared to their baseline (median number of bowel movements was 3 versus 1 at baseline [$P < 0.001$])

TABLE 2. Demographic and clinical data[a]

Patient characteristic	No. (%) or median (range)
Age (yr)	60 (21–96)
Female	76 (50.7%)
Race	
White	105 (70.0%)
Black	43 (28.7%)
Other	2 (1.4%)
Inflammatory bowel disease	13 (8.7%)
Abdominal surgery in previous 7 days	12 (8.0%)
Received laxative in previous 48 h	28 (18.7%)
Time from admission to stool collection (days)	2.9 (0–42.3)
Peak temp within 24 h of stool collection (°C)	37.1 (35.6–39.7)
Peak WBC count within 24 h of stool collection	8.8 (0.1–70.0)
Normal no. of bowel movements per day	1 (<1–9)
Normal stool consistency (BSC type)	3 (1–7)
Current no. of bowel movements per day	3 (<1–20)
Current stool consistency (BSC type)	7 (3–7)
Abdominal pain or cramping	1 (1–10)
Hyper- or hypoactive bowel sounds	28 (18.7%)
Abdominal tenderness	53 (35.3%)
Abdominal distention	20 (13.3%)
Involuntary guarding	20 (13.3%)
Rebound tenderness	30 (20.0%)
Clinically significant diarrhea	96 (64%)

[a] $n = 150$ patients. WBC, white blood cell; BSC, Bristol Stool Chart.

and were significantly more likely to have diarrhea compared to their baseline (median Bristol Stool Chart score was 7 versus 3 at baseline [$P < 0.001$]). Ninety-six patients (64%) met criteria for clinically significant diarrhea. Twenty-eight patients (19%) received a laxative in the 48 h prior to stool collection.

The results of the assay comparisons for standard A (i.e., positive toxigenic culture plus clinically significant diarrhea) are presented in Table 3. The cytotoxicity cell assay was the least sensitive (62.9%; 95% confidence interval [CI], 46.3% to 76.8%) in the comparison but was among the most specific (93.9%; 95% CI, 88.0% to 97.0%). Of the two toxin EIAs in the comparison, the Tox A/B from fresh stool was the least sensitive assay (80.0%; 95% CI, 64.1% to 90.0%) and the ProSpecT was the most sensitive (100%; 95% CI, 90.1% to 100%). Conversely, the most specific toxin EIA was the Tox A/B from fresh stool (93.9%; 95% CI, 88.0% to 97.0%) and the least specific was the ProSpecT (67.7%; 95% CI, 59.7% to 76.5%). All nucleic acid amplification tests, along with the Chek-60 GDH EIA, demonstrated superior sensitivity, but specificity was 86.1% or lower for these assays. A two-step algorithm of an initial screen with the Chek-60 GDH EIA resulted in a significant improvement in specificity for the ProSpecT ($P < 0.001$).

The prevalence of CDI in the first 100 specimens by standard A was 11%. The negative predictive value for all assays was >95% (Table 3). The positive predictive values of ProSpecT, Chek-60, and all three nucleic acid amplification tests were less than 50%. A GDH screen decreased the number of false positives for the nucleic acid amplification tests and ProSpecT, but the positive predictive value increased to above 50% only for the ProSpecT.

The results of the assay comparisons when the reference standards were having positive toxigenic culture while ignoring clinical presentation (standard B), having clinically significant diarrhea and at least four assays positive (standard C), and having at least four assays positive while ignoring clinical presentation (standard D) are provided in Tables 4, 5, and 6, respectively. The relative rankings of the assays by sensitivity and specificity within each comparison remain similar. The sensitivity of toxigenic culture and the ProSpecT relative to the other assays was lower when the laboratory component of the reference standard was "at least four assays positive."

The sensitivity of the assays was unchanged or decreased

TABLE 3. Assay comparison results with reference standard A[a]

Assay	No. of true-positive results ($n = 35$ specimens)	No. of true-negative results ($n = 115$ specimens)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
TechLab Tox A/B fresh	28	108	80.0 (64.1–90.0)	93.9 (88.0–97.0)	61.8 (39.8–78.8)	97.4 (95.2–98.7)
TechLab Tox A/B frozen	33	106	94.3 (81.4–98.4)	92.2 (85.8–95.8)	59.9 (41.5–74.3)	99.2 (97.4–99.8)
Remel ProSpecT frozen	35	79	100 (90.1–100)	67.7 (59.7–76.5)	28.3 (21.7–34.5)	100 (98.0–100)
Cytotoxicity from stool	22	108	62.9 (46.3–76.8)	93.9 (88.0–97.0)	56.0 (32.3–76.0)	95.3 (93.0–97.1)
TechLab Chek 60	35	93	100 (90.1–100)	80.9 (72.7–87.0)	39.3 (29.0–48.7)	100 (98.3–100)
BD GeneOhm	35	97	100 (90.1–100)	84.3 (76.6–89.9)	44.0 (32.2–55.0)	100 (98.4–100)
Cepheid	35	99	100 (90.1–100)	86.1 (78.6–91.3)	47.1 (34.2–58.7)	100 (98.5–100)
Illumigene	34	98	97.1 (85.5–99.5)	85.2 (77.6–90.6)	44.8 (32.1–56.7)	99.6 (97.7–99.9)
Toxigenic culture	35	106	100 (90.1–100)	92.2 (85.8–95.8)	61.3 (44.0–74.6)	100 (98.6–100)
GDH then TechLab Tox A/B frozen	33	106	94.3 (81.4–98.4)	92.2 (85.8–95.8)	59.9 (41.5–74.3)	99.2 (97.4–99.8)
GDH then Remel ProSpecT frozen	35	103	100 (90.1–100)	89.6 (82.6–93.9)	54.3 (39.0–67.0)	100 (98.5–100)
GDH then cytotoxicity from stool	22	109	62.9 (46.3–76.8)	94.8 (89.1–97.6)	59.9 (34.4–79.8)	95.4 (93.1–97.1)
GDH then BD GeneOhm	35	100	100 (90.1–100)	87.0 (79.6–91.9)	48.7 (35.5–60.4)	100 (98.5–100)
GDH then Cepheid	35	100	100 (90.1–100)	87.0 (79.6–91.9)	48.7 (35.3–60.4)	100 (98.5–100)
GDH then Illumigene	34	101	97.1 (85.5–99.5)	87.8 (80.1–92.6)	49.6 (34.7–62.4)	99.6 (97.8–99.9)
GDH then toxigenic culture	35	106	100 (90.1–100)	92.2 (85.8–95.8)	61.3 (44.0–74.6)	100 (98.6–100)

[a] Shown are assay comparison results with reference standard A (stool samples positive by toxigenic culture plus the presence of clinically significant diarrhea). The prevalence for positive predictive value (PPV) and negative predictive value (NPV) calculations was 11%. If samples were positive by a screen with glutamate dehydrogenase EIA (GDH; TechLab Chek-60), the final result was positive only if the second assay was also positive. PPV and NPV estimates were calculated with the CDI prevalence for the corresponding reference standard and point estimates of sensitivity and specificity. Fresh, assay using fresh stool sample; frozen, assay using frozen stool sample.

TABLE 4. Assay comparison results with reference standard B[a]

Assay	No. of true-positive results (n = 44 specimens)	No. of true-negative results (n = 106 specimens)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
TechLab Tox AB fresh	33	104	75.0 (60.6–85.4)	98.1 (93.4–99.5)	84.3 (55.6–95.9)	96.6 (94.6–98.0)
TechLab Tox AB frozen	39	103	88.6 (76.0–95.1)	97.2 (92.0–99.0)	81.2 (56.2–92.8)	98.4 (96.6–99.3)
Remel ProSpecT frozen	44	79	100 (92.0–100)	74.5 (65.5–81.9)	34.8 (26.7–43.0)	100 (98.4–100)
Cytotoxicity from stool	28	105	63.6 (48.9–76.2)	99.1 (94.9–99.8)	90.6 (56.7–98.1)	95.2 (93.2–96.9)
TechLab Chek 60	44	93	100 (92.0–100)	87.7 (80.1–92.7)	52.6 (38.7–65.1)	100 (98.7–100)
BD GeneOhm	43	96	97.7 (88.2–99.6)	90.6 (83.5–94.8)	58.6 (42.2–72.3)	99.7 (98.1–99.9)
Cepheid	44	99	100 (92.0–100)	93.4 (87.0–96.8)	67.4 (49.1–81.0)	100 (98.8–100)
Illumigene	42	97	95.5 (84.9–98.7)	91.5 (84.7–95.5)	60.5 (43.1–74.9)	99.3 (97.6–99.8)
Toxigenic culture	44	106	100 (92.0–100)	100 (96.5–100)	100 (78.2–100)	100 (98.9–100)
GDH then TechLab Tox AB frozen	39	103	88.6 (76.0–95.1)	97.2 (92.0–99.0)	81.2 (56.4–92.8)	98.4 (96.6–99.3)
GDH then Remel ProSpecT frozen	44	103	100 (92.0–100)	97.2 (92.0–99.0)	83.0 (61.1–93.2)	100 (98.8–100)
GDH then cytotoxicity from stool	28	106	63.6 (48.9–76.2)	100 (96.5–100)	100 (65.6–100)	95.3 (93.3–96.9)
GDH then BD GeneOhm	43	99	97.7 (88.2–99.6)	93.4 (87.0–96.8)	66.9 (48.1–80.9)	99.7 (98.2–99.9)
GDH then Cepheid	44	100	100 (92.0–100)	94.3 (88.2–97.4)	70.5 (51.5–84.0)	100 (98.9–100)
GDH then Illumigene	42	100	95.5 (84.9–98.7)	94.3 (88.2–97.4)	69.6 (49.5–83.8)	99.4 (97.7–99.8)
GDH then toxigenic culture	44	106	100 (92.0–100)	100 (96.5–100)	100 (78.2–100)	100 (98.9–100)

[a] Shown are assay comparison results with reference standard B (stool samples positive by toxigenic culture only). The prevalence for positive predictive value (PPV) and negative predictive value (NPV) calculations was 12%. If samples were positive by a screen with glutamate dehydrogenase EIA (GDH; TechLab Chek-60), the final result was positive only if the second assay was also positive. PPV and NPV estimates were calculated with the CDI prevalence for the corresponding reference standard and point estimates of sensitivity and specificity. Fresh, assay using fresh stool sample; frozen, assay using frozen stool sample.

with the alternate reference standards compared to standard A. The only significant change was for toxigenic culture when the reference standard was at having least four positive assays while ignoring clinical presentation (standard D) (P = 0.04). The specificity and positive predictive values of all of the assays increased with standards B through D versus those for standard A. There were trends or significant increases in specificity when standard B (having toxigenic culture while ignoring clinical presentation) was the reference standard for Tox A/B from a frozen specimen, the cytotoxicity cell assay, Cepheid ($0.1 \geq P > 0.05$ for all three), and toxigenic culture (P = 0.004). When the reference standard was having at least four assays positive while ignoring clinical presentation (standard D), there were significant increases in specificity for Tox A/B from fresh and frozen stool, Chek-60, BD GeneOhm, Cepheid, Illumigene, and toxigenic culture (P was <0.01 for all except Tox A/B from fresh stool, for which P was 0.016) compared that to when standard A was the reference standard.

There were 15 patients whose stool specimen was reported as negative for *C. difficile* toxins (Tox A/B from fresh stool) but

TABLE 5. Assay comparison results with reference standard C[a]

Assay	No. of true-positive results (n = 40 specimens)	No. of true-negative results (n = 110 specimens)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
TechLab Tox AB fresh	30	105	75.0 (59.8–85.8)	95.5 (89.8–98.0)	73.1 (48.8–87.5)	95.9 (93.2–97.7)
TechLab Tox AB frozen	36	104	90.0 (77.0–96.0)	94.6 (88.6–97.5)	72.7 (52.4–86.2)	98.3 (95.9–99.3)
Remel ProSpecT frozen	37	76	92.5 (80.1–97.4)	69.1 (59.9–77.0)	32.8 (24.5–40.8)	98.3 (94.9–99.5)
Cytotoxicity from stool	22	103	55.0 (39.8–69.3)	93.6 (87.4–96.9)	58.3 (34.0–78.4)	92.7 (89.9–95.1)
TechLab Chek 60	40	93	100 (91.2–100)	84.6 (76.6–90.1)	51.2 (38.8–62.2)	100 (98.2–100)
BD GeneOhm	40	97	100 (91.2–100)	88.2 (80.8–93.0)	58.0 (43.6–69.9)	100 (98.3–100)
Cepheid	40	99	100 (91.2–100)	90.0 (83.0–94.3)	61.9 (46.6–74.1)	100 (98.3–100)
Illumigene	39	98	97.5 (87.1–99.6)	89.1 (81.9–93.7)	59.3 (43.9–72.0)	99.5 (97.5–99.9)
Toxigenic culture	35	101	87.5 (73.9–94.5)	91.8 (85.2–95.6)	63.5 (44.8–77.8)	97.8 (95.2–99.1)
GDH then TechLab Tox AB frozen	36	104	90.0 (77.0–96.0)	94.6 (88.6–97.5)	72.7 (52.4–86.2)	98.3 (95.9–99.3)
GDH then Remel ProSpecT frozen	37	100	92.5 (80.1–97.4)	90.9 (84.1–95.0)	62.3 (45.1–76.0)	98.7 (96.3–99.6)
GDH then cytotoxicity from stool	22	104	55.0 (39.8–69.3)	94.6 (88.6–97.5)	61.9 (36.2–81.9)	92.8 (90.0–95.1)
GDH then BD GeneOhm	40	100	100 (91.2–100)	90.9 (84.1–95.0)	64.1 (48.3–76.5)	100 (98.3–100)
GDH then Cepheid	40	100	100 (91.2–100)	90.9 (84.1–95.0)	64.1 (48.3–76.5)	100 (98.3–100)
GDH then Illumigene	39	101	97.5 (87.1–99.6)	91.8 (85.2–95.6)	65.9 (48.9–78.7)	99.6 (97.6–99.9)
GDH then toxigenic culture	35	101	87.5 (73.9–94.5)	91.8 (85.2–95.6)	63.5 (44.8–77.8)	97.8 (95.2–99.1)

[a] Shown are assay comparison results with reference standard C (stool samples positive by at least four assays from frozen specimens plus the presence of clinically significant diarrhea). The prevalence for positive predictive value (PPV) and negative predictive value (NPV) calculations was 14%. If samples were positive by a screen with glutamate dehydrogenase EIA (GDH; TechLab Chek-60), the final result was positive only if the second assay was also positive. PPV and NPV estimates were calculated with the CDI prevalence for the corresponding reference standard and point estimates of sensitivity and specificity. Fresh, assay using fresh stool sample; frozen, assay using frozen stool sample.

TABLE 6. Assay comparison results with reference standard D[a]

Assay	No. of true-positive results (n = 50 specimens)	No. of true-negative results (n = 100 specimens)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
TechLab Tox AB fresh	35	100	70.0 (56.3–80.1)	100 (96.3–100)	100 (74.3–100)	94.6 (92.0–96.3)
TechLab Tox AB frozen	42	100	84.0 (71.5–91.7)	100 (96.3–100)	100 (78.6–100)	97.0 (94.7–98.4)
Remel ProSpecT frozen	47	76	94.0 (83.8–97.9)	76.0 (66.8–83.3)	42.7 (32.5–52.8)	98.5 (95.6–99.5)
Cytotoxicity from stool	28	99	56.0 (42.3–68.8)	99.0 (94.6–99.8)	91.4 (59.9–98.5)	92.2 (89.6–94.4)
TechLab Chek 60	50	93	100 (92.9–100)	93.0 (86.3–96.6)	73.1 (56.4–84.9)	100 (98.5–100)
BD GeneOhm	49	96	98.0 (89.5–99.7)	96.0 (90.2–98.4)	82.4 (63.5–92.2)	99.6 (97.8–99.9)
Cepheid	49	98	98.0 (89.5–99.7)	98.0 (93.0–99.5)	90.3 (70.9–97.4)	99.6 (97.9–99.9)
Illumigene	48	97	96.0 (86.5–98.9)	97.0 (91.6–99.0)	85.9 (66.2–95.0)	99.2 (97.3–99.8)
Toxigenic culture	44	100	88.0 (76.2–94.4)	100 (96.3–100)	100 (79.7–100)	97.8 (95.5–98.9)
GDH then TechLab Tox AB frozen	42	100	84.0 (71.5–91.7)	100 (96.3–100)	100 (78.6–100)	97.0 (94.7–98.4)
GDH then Remel ProSpecT frozen	47	100	94.0 (83.8–97.9)	100 (96.3–100)	100 (81.2–100)	98.9 (96.9–99.6)
GDH then cytotoxicity from stool	28	100	56.0 (42.3–68.8)	100 (96.3–100)	100 (68.5–100)	92.3 (89.9–94.4)
GDH then BD GeneOhm	49	99	98.0 (89.5–99.7)	99.0 (94.6–99.8)	94.9 (75.9–99.0)	99.6 (97.9–99.9)
GDH then Cepheid	49	99	98.0 (89.5–99.7)	99.0 (94.6–99.8)	94.9 (75.9–99.0)	99.6 (97.9–99.9)
GDH then Illumigene	48	100	96.0 (86.5–98.9)	100 (96.3–100)	100 (81.7–100)	99.2 (97.4–99.8)
GDH then toxigenic culture	44	100	88.0 (76.2–94.4)	100 (96.3–100)	100 (79.7–100)	97.8 (95.5–98.9)

[a] Shown are assay comparison results with reference standard D (stool samples positive by at least four assays only). The prevalence for positive predictive value (PPV) and negative predictive value (NPV) calculations was 16%. If samples were positive by a screen with glutamate dehydrogenase EIA (GDH; TechLab Chek-60), the final result was positive only if the second assay was also positive. PPV and NPV estimates were calculated with the CDI prevalence for the corresponding reference standard and point estimates of sensitivity and specificity. Fresh, assay using fresh stool sample; frozen, assay using frozen stool sample.

was positive by at least four assays (Table 7). Eleven (73.3%) of these patients were positive by toxigenic culture, and three (20%) were positive by the cytotoxicity cell assay. Ten (66.7%) had clinically significant diarrhea. Five (33.3%) of the patients whose Tox A/B result from fresh stool was discordant with the "at least four assays positive" criteria received a laxative in the 48 h prior to stool specimen collection compared to 23 patients (17.0%) whose Tox A/B result from fresh stool was concordant (P = 0.16). Four patients (26.7%) received empirical treatment for CDI. Three patients died from reasons unrelated to CDI. None of the 15 patients were subsequently diagnosed with CDI.

TABLE 7. Treatment and outcomes of patients whose *C. difficile* test was reported as negative by Techlab Tox A/B from fresh specimen but was positive by at least four assays

Subject identification no.	Clinically significant diarrhea	Laxative use	Empirical CDI treatment	Died in 60-day follow-up	Diagnosed with CDI in 60-day follow-up
15[a]	Yes	Yes	No	No	No
21[a]	Yes	No	Metronidazole	Yes[d]	No
25	No	No	No	No	No
35[b]	Yes	Yes	No	No	No
54[b]	Yes	No	No	Yes[e]	No
70[b]	Yes	Yes	No	No	No
82[a,c]	Yes	No	Metronidazole	No	No
109[a]	No	No	No	No	No
112[a]	No	No	No	No	No
113[a,c]	Yes	No	No	No	No
118[a,c]	No	No	No	No	No
122[a]	Yes	No	Vancomycin	No	No
126[a]	No	Yes	Metronidazole	No	No
148[a]	Yes	No	No	No	No
149[a]	Yes	Yes	No	Yes[f]	No

[a] Toxigenic *C. difficile* isolated from stool.
[b] Nontoxigenic *C. difficile* isolated from stool.
[c] Cytotoxicity cell assay of stool was positive.
[d] Patient died from ischemic cardiomyopathy.
[e] Patient died from respiratory failure secondary to metastatic carcinoma.
[f] Patient died from pneumonia.

DISCUSSION

The *C. difficile* diagnostic assay literature suffers from use of different reference standards, inconsistent reporting of stool collection criteria, and most importantly, a lack of data on severity of diarrhea and CDI-related outcomes. Asymptomatic *C. difficile* colonization is common in patient populations that are tested for *C. difficile* (3, 16). Therefore, data on the patient's clinical presentation are needed for accurate interpretation of assay results for the diagnosis of CDI (3, 14). To our knowledge, there is only one other *C. difficile* assay comparison in the literature that included a prospective patient evaluation of patients who had stools submitted for *C. difficile* testing based on orders from a treating clinician (13). Peterson et al. found the sensitivity, specificity, and positive and negative predictive values of their in-house nucleic acid amplification test to be 93.3%, 97.4%, 75.7%, and 99.4%, respectively. Thirty-nine percent of patients did not meet their criteria for clinically significant diarrhea, similar to the 36% in this study. However, they excluded the stools from these patients in their comparison. This likely biased the specificity of the assays in their comparison to be higher than if these patients were not excluded. Therefore, our study, by including patients whose treating clinicians ordered a test for *C. difficile* but who did not have clinically significant diarrhea, is more generalizable to the population of hospitalized patients being tested for *C. difficile.* The results of this study support the hypothesis that including clinical presentation in the reference standard will decrease the specificity of *C. difficile* assays for the diagnosis of CDI. Although limited by small sample size, the outcome data collected support this hypothesis, i.e., there were no CDI-related

adverse events in the patients whose assay result was reported as negative (*C. difficile* Tox A/B II from fresh stool) but found to be positive by other assays.

When clinical criteria were included in the reference standard, the positive predictive value of some assays decreased such that there would be as many, if not more, false positives as true positives (Tables 3 through 6). While it is important to accurately diagnose true cases of CDI to avoid delays in treatment and the risk of disease progression, there are likely costs due to false-positive results as well. In one placebo-controlled study of treating asymptomatic *C. difficile* carriers, the only patient to develop CDI after treatment was stopped had received oral vancomycin (8). Other potential costs include direct and indirect costs of placing patients under contact precautions when not otherwise indicated, decreases in patient satisfaction, and increases in adverse events associated with contact precautions (10, 17, 21). Increases in CDI incidence due to false-positive assays will also divert limited infection prevention and control resources and lead to false impressions if CDI rates are reported to the public.

The appropriate reference standard when comparing *C. difficile* assays is not clear. The Society for Healthcare Epidemiology of America and Infectious Diseases Society of America guidelines recommend toxigenic culture as the gold standard laboratory comparator (B-III recommendation) (3). Toxigenic *C. difficile* was not isolated from six specimens that were positive by at least four assays in this study. *C. difficile* was cultured from five of these specimens, but cytotoxicity was not detected from culture. These patients may have had more than one strain of *C. difficile* present, and the isolates selected to assess toxin production were nontoxigenic. Interestingly, four of these strains were positive for the *tcdB* toxin B gene. The optimal methods for detecting toxin production from culture have not been defined (15, 19). It is possible that our method of performing the cytotoxicity cell assay was not sufficiently sensitive to detect toxin production. Because of the discordance between cytotoxicity testing and the presence of the *tcdB* gene, cytotoxicity testing of these isolates was repeated with Vero cells, and the incubation period was extended to 96 h for both cell lines. Cytotoxicity was still not identified. Two of these patients had a negative Tox A/B result for fresh specimen and were never treated for CDI (Table 7, subjects 35 and 70). Subject 70 also had a colonoscopy, which was normal. The other two patients had other potential explanations for diarrhea and had a very protracted improvement in symptoms after CDI treatment was started, suggesting that the diarrhea was not due solely to CDI. These unique strains are being further characterized, by methods including whole-genome sequencing, to determine if the lack of cytotoxicity is due to a decrease in toxin production or production of nonfunctional toxin.

Some investigators feel that toxigenic culture is too sensitive and may detect asymptomatic carriage in patients with diarrhea for other reasons and that the cytotoxicity cell assay is the most appropriate reference standard for identifying patients with CDI because it detects biologically active toxin (15). Notably, the cytotoxicity cell assay was the least sensitive method in this study. This may be due to degradation of toxin in the specimen during transport or upon prolonged storage or methodological problems with the assay. However, the assay was performed using methods previously described by an investigator experienced in the technique (2). The performance of the assay may be related to poor patient selection for *C. difficile* testing. Conversely, the 95% confidence interval of the cytotoxicity cell assay overlaps with the 95% confidence intervals of the Tox A/B and was within the ranges of reported sensitivities in the literature (3, 22). It may have been the least sensitive assay in this study by chance as well (22).

Some *C. difficile* assay comparisons use a nontoxigenic culture, noncytotoxicity cell assay reference standard, or composite endpoint where true positives are specimens that are positive by multiple assays (9, 11, 13, 18). Due to the difficulties of performing toxigenic culture and the cytotoxicity cell assay, this is frequently the reference standard that clinical laboratories use when conducting assay comparisons and, hence, the reason a composite laboratory standard was assessed here. There are potential problems with this approach. The reference standard is a surrogate for the desired endpoint (toxigenic *C. difficile* or the presence of active toxin in stool). In addition, as demonstrated in this study, a composite endpoint biases the results toward improved sensitivity and specificity of the assays included in the composite endpoint.

There were other interesting findings in this study. Although the Techlab assays warn of a possible decrease in sensitivity with freezing, the Tox A/B from the frozen specimen was more sensitive than that from the fresh specimen, and the Chek-60 had 100% sensitivity. The Tox A/B from the frozen specimen was performed by a single experienced medical technologist as a part of this study. The Tox A/B from the fresh sample was performed as part of a constellation of duties by a technologist on the third shift. Other studies do indicate there may be variability in toxin EIA sensitivities across laboratories (5, 12, 22). This may be due to operator variability. Notably, almost 20% of patients were on a laxative. This is indicative of poor patient selection for *C. difficile* testing and may have impacted our findings. The least specific assay was the ProSpecT. This was consistent with the results of the epidemiological investigation that was performed when a dramatic drop in CDI incidence was noted after the assay at our institution was changed to the Tox A/B (7).

This study demonstrates the importance of clinical information when interpreting *C. difficile* assay results. The high proportion of patients without clinically significant diarrhea and the number of patients on laxatives indicate that we need validated criteria for when to test for *C. difficile*. Another potential area of study is validation of inflammatory biomarkers to help determine when detection of toxigenic *C. difficile* from stool is clinically significant (1, 20). Finally, there is an urgent need for *C. difficile* assay comparison studies that are adequately powered to assess patient outcomes. Although there has been great enthusiasm in the literature and the clinical microbiology community regarding the use of molecular diagnostics for the diagnosis of CDI, the optimal method for diagnosing CDI will remain elusive until these issues are resolved.

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