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Multicenter Evaluation of a New Screening Test That Detects *Clostridium difficile* in Fecal Specimens

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***Clostridium difficile* causes approximately 25% of nosocomial antibiotic-associated diarrheas and most cases of pseudomembranous colitis. We evaluated *C. DIFF CHEK*, a new screening test that detects glutamate dehydrogenase of *C. difficile*. Our results showed that this test was comparable to PCR in sensitivity and specificity and outperformed bacterial culture.**

Clostridium difficile causes approximately 25% of the antibiotic-associated diarrheas (AAD) and most cases of pseudomembranous colitis (8, 21). In the United States, there are an estimated 300,000 cases of nosocomial *C. difficile*-associated diarrhea and colitis every year, resulting in an annual economic burden of over one billion dollars to the health care system (7). The disease results from the two toxins, A and B, produced by toxigenic strains (8). Typical toxigenic strains (designated A^+/B^+) produce both toxins at comparable levels (11, 15). Atypical toxigenic strains (designated A^-/B^+) produce only toxin B. Nontoxigenic strains (A^-/B^-) do not carry the pathogenicity locus that houses *toxA* and *toxB* and three other, smaller open reading frames, do not produce toxin, and are nonpathogenic (20). Recently, researchers have identified mutations and deletions in the *toxA* and *toxB* genes, resulting in highly complicated toxinotypes (14, 17, 18). The enzyme glutamate dehydrogenase (GDH), a metabolic enzyme consisting of six identical subunits (M_r of 43,000), is expressed at high levels by all strains of *C. difficile* and is referred to as the common antigen (23).

The diagnosis of *C. difficile* disease typically is based on a clinical history of recent antibiotic usage and diarrhea in combination with in vitro laboratory tests for *C. difficile* toxin or GDH (23). Tests for toxin include tissue culture assay, enzyme-linked immunosorbent assay (ELISA), and membrane tests. Tissue culture assay is considered by many to represent the "gold standard" because of its sensitivity. However, tissue culture assay is tedious and time consuming, with a turnaround time of 48 h before a specimen can be ruled negative. Although less sensitive than tissue culture, ELISA and membrane tests are popular because they give results within minutes or hours and some results can be determined visually. Currently, PCR is used only as a research tool in laboratories for detecting *C. difficile* toxin genes in fecal specimens (3, 5, 6).

Many laboratories utilize screening tests that detect the presence of *C. difficile* in fecal specimens, either by tests that target GDH or by bacterial culture (2, 4). None of these screening tests specifically identifies toxigenic strains, since GDH is produced by toxigenic (both typical A^+/B^+ and atypical A^-/B^+) and nontoxigenic A^-/B^- strains. The early GDH-based tests suffered from lower sensitivity and specificity and cross-reacted with other colonic bacteria (9, 13). Even so, GDH has proven to be a good marker for the organism (12) because it is produced in relatively large amounts by all *C. difficile* strains. In the study described here, we evaluated a new screening ELISA test, the *C. DIFF CHEK*, by comparing it to a sensitive in-house PCR assay for the GDH gene *gluD* and to bacterial culture. As an additional measurement of performance, we identified toxin-positive samples by tissue culture assay.

Fecal specimens were supplied to TechLab (Blacksburg, Va.; site 1) from hospital laboratories, to Providence Portland Medical Center (Portland, Ore.; site 2), or to TriCore Reference Laboratories (Albuquerque, N.M.; site 3). All specimens were submitted for routine *C. difficile* toxin testing because of reported AAD. The *C. DIFF CHEK* test (TechLab), bacterial culture, and tissue culture assay were performed on site unless noted. Aliquots of fecal samples were sent to TechLab for PCR. All samples were stored at 2 to 8°C for up to 72 h or were frozen and thawed no more than twice prior to analysis. Study plans were reviewed and approved by appropriate Institutional Review Board committees at the individual institutions where the samples were collected. All samples were coded prior to testing to protect patient identity.

Two formats, *C. DIFF CHEK-60* and *C. DIFF CHEK-30*, were evaluated. *C. DIFF CHEK-60* requires a 50-min incubation at 37°C followed by a 10-min substrate incubation at room temperature. *C. DIFF CHEK-30* requires a 20-min incubation at 37°C in a shaking incubator followed by a 10-min substrate incubation. Both formats were used according to the manufacturer's instructions.

Bacterial culture at site 2 was performed by applying 1 μl of fresh fecal specimen onto selective cycloserine–cefoxitin-fructose agar (CCFA) (REMEL, Lenexa, Kans.), followed by incubation under anaerobic conditions for 48 to 72 h at 37°C. When the colonies were clustered or questionable, repeat plating was performed. Presumptive isolates were inoculated into cooked meat broth (REMEL), and the medium was incubated anaerobically for 48 h at 37°C. Aliquots of chopped meat culture were used to inoculate prereduced blood agar plates. All isolates were Gram stained and further identified with the

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TABLE 1. Comparison of the *C. DIFF CHEK-60, C. DIFF CHEK-30,* PCR for GDH gene *gluD,* and bacterial culture for detecting *C. difficile* in fecal specimens

Total no. (%) of samples with profile[a]	Presence of *C. difficile* determined by:				No. (%) of samples with profile for site[c]:		
	C. DIFF CHEK-60[b]	*C. DIFF CHEK-30*[b]	PCR for *gluD*	Bacterial culture	1[d]	2[e]	3[f]
114 (11.5)	+	+	+	+	116 (7.5)	28 (5.8)	70 (23.3)
689 (69.5)	−	−	−	−	146 (68.9)	374 (77.9)	169 (56.3)
73 (7.4)	+	+	+	−	15 (7.1)	41 (8.5)	17 (5.7)
46 (4.6)	−	−	−	+	25 (11.8)	2 (0.4)	19 (6.3)
19 (1.9)	−	−	+	−	5 (2.4)	12 (2.5)	2 (0.7)
14 (1.4)	+	+	−	−	2 (0.9)	6 (1.3)	6 (2.0)
13 (1.3)	+	−	−	−	2 (0.9)	5 (1.0)	6 (2.0)
10 (1.0)	−	+	−	−	0 (0.0)	7 (1.5)	3 (1.0)
5 (0.5)	−	+	+	−	1 (0.5)	3 (0.6)	1 (0.3)
3 (0.3)	+	−	−	+	0 (0.0)	0 (0.0)	3 (1.0)
3 (0.3)	−	−	+	+	0 (0.0)	1 (0.2)	2 (0.7)
2 (0.2)	+	+	−	+	0 (0.0)	1 (0.2)	1 (0.3)
1 (0.1)	+	−	+	−	0 (0.0)	0 (0.0)	1 (0.3)

[a] Of 992 samples tested, 796 were from patients whose sex was recorded. Included were 308 samples (39%) from male patients and 488 samples (61%) from female patients. The age range for patients included in the study was 8 months to 96 years. Stool samples from babies (8 months to 2 years) were not excluded from this study because only the presence of *C. difficile* and its toxins were tested and the test results were not linked to the diagnosis of *C. difficile* disease. Fecal samples were of different consistencies, including liquid (37%), semisolid or soft (45%), and solid or formed (18%).

[b] Although the results of the *C. DIFF CHEK* test can be measured visually, by single wavelength at 450 nm, and by dual wavelength at 450 and 620 nm, only the dual-wavelength measurements were evaluated for this study.

[c] Each sample was tested once using each assay. In some instances, multiple samples from the same patients may have been involved.

[d] TechLab (n = 212).

[e] Providence Portland Medical Center (n = 480).

[f] TriCore Reference Laboratories (n = 300).

RapID ANA II System (REMEL). At sites 1 and 3, approximately 100 μl of fecal specimen was plated onto CCFA (Anaerobe Systems, Morgan Hill, Calif.), and the inoculated plates were incubated at 37°C anaerobically for 48 to 72 h. Presumptive *C. difficile* colonies on CCFA were characterized by their large size (about 4 mm), yellow color, ground-glass appearance, circular shape with slight filamentous edge, low umbonate to flat in profile, and horsy smell (22). Specimens were tested for cytotoxic activity using the *TOX B TEST* (TechLab).

For PCR, DNA was extracted from fecal material using the QIAamp DNA stool mini kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions and kept frozen until use. PCR was performed using Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, N.J.) with 15 μl of primers, each at a concentration of 1.67 μM, and 10 μl of extracted fecal DNA. The PCR for the GDH gene, *gluD*, was carried out using 45 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. Primers were designed based on *C. difficile gluD* sequence (10) and included GGAAAAGATGTAAATGTCTT CGAGATG and CTGATTTACACCATTCAGCCATAGC. The presence of a 0.75-kb *gluD* gene amplicon was determined electrophoretically on a 1% agarose gel. In each assay, aliquots (1 ng) of DNA from *C. difficile* VPI 11186 or water were used as positive and negative controls, respectively. The minimal amount of DNA detected was 1 copy of *gluD* gene per reaction, which is equivalent to about 100 copies per ml of feces. Discrepant samples with negative PCR results, along with more than 50 negative samples, were checked for PCR inhibition. No PCR inhibition was observed with these samples.

A total of 992 fecal samples were analyzed by the 30-min and 60-min *C. DIFF CHEK* tests, PCR for the *gluD* gene and by bacterial culture (Table 1). There were 114 samples (12%) positive and 689 samples (70%) negative by all four tests. The remaining 189 samples gave discrepant results among the four tests. Within the discrepant group, 73 samples (7%) were positive by the *C. DIFF CHEK* test and PCR but negative by bacterial culture. In general, the two formats of the *C. DIFF CHEK* test exhibited a high correlation with each other and with PCR. When compared to each other, the *C. DIFF CHEK* formats exhibited a correlation of 97% (P value of 0.7237 by McNemar's test [19], which assesses the significant similarity between two tests). When compared to PCR, the 30-min format exhibited a correlation of 95% (P value of 0.6682), and the 60-min format had a correlation of 94% (P value of 0.5151). Bacterial culture, on the other hand, exhibited a considerably lower correlation with the two *C. DIFF CHEK* formats and PCR. The correlation of bacterial culture with either format was 85% (P value of <0.0001). The correlation of bacterial culture with PCR was 86% (P value of <0.0001). These findings demonstrated that the *C. DIFF CHEK* formats and PCR performed similarly. Bacterial culture, on the other hand, was significantly different from these assays. Bacterial culture also gave more variation than the other tests, as noted by the lower isolation rate observed at site 2. Lab-to-lab variation in bacterial culture has been reported previously (16).

An analysis of each of the screening tests compared to tissue culture assay is presented in Table 2. There were 137 samples (14%) that were positive by tissue culture assay. Both formats of the *C. DIFF CHEK* and PCR exhibited sensitivities of 93% or higher and specificities of 89% or higher when compared to tissue culture. The positive predictive values, while only about 58% or higher for these three assays, included samples that contained nontoxigenic strains that are negative in the tissue culture assay. The three tests each exhibited negative predictive values of ca. 99%. Bacterial culture exhibited a sensitivity (64%) and negative predictive value (94%) that were significantly lower than those of the other tests. When tissue culture assay-positive results were used as a reference parameter, McNemar's test (19) exhibited a P value of 0.3173 for the two

TABLE 2. Comparison of the *C. DIFF CHEK-60, C. DIFF CHEK-30,* PCR for *gluD,* and bacterial culture to tissue culture assay

Test	Result	No. of samples with tissue culture assay result[a]		Sensitivity (%)	Specificity (%)	PPV (%)[b]	NPV (%)[c]	Correlation (%)
		Positive	Negative					
C. DIFF CHEK-60	Positive	127	93	92.7	89.1	57.7	98.7	89.6
	Negative	10	762					
C. DIFF CHEK-30	Positive	129	89	94.2	89.6	59.2	99.0	90.2
	Negative	8	766					
PCR for *gluD*	Positive	127	88	92.7	89.7	59.1	98.7	90.1
	Negative	10	767					
Bacterial culture	Positive	87	81	63.5	90.5	51.8	93.9	86.8
	Negative	50	774					

[a] Tissue culture assay for site 3 was performed at TechLab.
[b] Positive predictive value.
[c] Negative predictive value.

formats of *C. DIFF CHEK*, 1.0000 for *C. DIFF CHEK-60* with PCR, and 0.4795 for *C. DIFF CHEK-30* with PCR. On the other hand, using the same approach, the *P* values for bacterial culture with any of these three tests were less than 0.0001, demonstrating lower correlation of bacterial culture with tissue culture assay.

Compared to tissue culture assay, the GDH-based tests studied here exhibited high sensitivity and low specificity, which was reported by other investigators with the recent and more sensitive GDH-based tests (1, 20). In our study, we found that only about 60% of the GDH-positive samples were positive by tissue culture assay. We cannot rule out the possibility that some of the remaining 40% of negative specimens were true positives that contained amounts of toxin below the detection limits of the tissue culture assay. Therefore, although a positive GDH and negative toxin result may be indicative of a nontoxigenic strain, these results should alert the physician to monitor the patient closely and to perform additional testing if necessary.

A low percentage of specimens (ca. 1%) were negative by the *C. DIFF CHEK* and PCR but positive by tissue culture assay, a phenomenon reported previously by others (1). *C. DIFF CHEK* and PCR-*gluD* are specific assays that do not react with either *Clostridium sordellii* or the other bacteria that caused cross-reactivity in the earlier GDH-based tests. However, the toxins from *C. sordellii* cross-react with the antibody against *C. difficile* toxins, which might explain our observation. This phenomenon might also be explained by degradation of GDH in fecal specimens. However, based on the low percentage of samples that are in this category, the susceptibility of GDH to factors such as degradation by fecal proteases does not appear to be a primary concern. Degradative enzymes (e.g., DNase) may also help to explain why some specimens (ca. 1%) were negative by PCR but positive by the *C. DIFF CHEK* and tissue culture assay.

C. difficile disease represents a complicated process primed by the disturbance of normal flora by antibiotic treatment. The *C. DIFF CHEK* is a new test that offers clinical utility as a screen for patients suspected of having *C. difficile* disease. The *C. DIFF CHEK* targets GDH as a marker antigen, but utilizes an ELISA format. Our results showed that both the 30-min and 60-min formats performed similarly to a PCR assay for the GDH gene *gluD*. Both exhibited negative predictive values of roughly 99%, supporting their value for ruling specimens negative for *C. difficile*. Like other GDH tests, this test does not confirm the presence of toxigenic strains in patients. A toxin-specific test must be performed to verify the presence of toxigenic *C. difficile*. However, clinical laboratories that utilize a time-consuming toxin-confirmatory test, such as tissue culture assay or bacterial culture, may consider GDH-based screens to minimize the number of specimens that require confirmatory testing. This approach may make laboratory testing for *C. difficile* more efficient by reducing health care costs and minimizing unnecessary treatment with metronidazole or vancomycin.

In summary, our results showed that the *C. DIFF CHEK* and PCR outperformed bacterial culture, suggesting that the *C. DIFF CHEK* test is a more accurate indicator than culture for detecting *C. difficile* in a fecal specimen. The PCR assay that we used as an aid in the evaluation of the *C. DIFF CHEK* test is highly sensitive, detecting ca. 1 copy of a control plasmid that carries the *gluD* gene of *C. difficile*. Thus, the *C. DIFF CHEK* test matches the high sensitivity of PCR but offers the clinical laboratory a simpler, faster, and more cost-effective screening test for *C. difficile*.

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