An Evaluation of tcdB Real-Time PCR in a Three-Step Diagnostic Algorithm for the Detection of Toxigenic Clostridium difficile

Ann M. Larson¹,², Angela M. Fung³ and Ferric C. Fang¹,²,³*

¹Clinical Microbiology Laboratory
Harborview Medical Center
Seattle, WA 98104

²Departments of Laboratory Medicine and ³Microbiology
University of Washington School of Medicine
Seattle, WA 98195

*Corresponding Author: Department of Microbiology, University of Washington, Box 357242, Seattle, WA 98195-7242 USA, Phone: 206-221-6770, Fax: 206-616-1575, E-mail: fcfang@u.washington.edu
ABBREVIATIONS: AB-Q, QUIK CHEK toxin A/B EIA; CD, *Clostridium difficile*; CDI, *Clostridium difficile* infection; CYT, cytotoxin assay by tissue culture; DPCR, direct polymerase chain reaction; GDH-Q, QUIK CHEK C. *difficile* glutamate dehydrogenase EIA; tcdA, toxin A gene; tcdB, toxin B gene; tcdC, gene encoding negative regulator of toxin expression.
Clostridium difficile (CD) is the most common infectious cause of diarrhea in hospitalized patients. The optimal approach for the detection of toxigenic CD remains controversial because no single test is sensitive, specific and affordable. We have developed a real-time PCR method (DPCR) to detect the \textit{tcdB} gene encoding toxin B directly from stool specimens and have combined it with enzyme immunoassays in a three-step protocol. DPCR was performed on 699 specimens that were positive for CD glutamate dehydrogenase by Wampole C DIFF QUIK CHEK EIA (GDH-Q) and negative for toxins A and B by Wampole TOX A/B QUIK CHEK EIA (AB-Q), performed sequentially. The performance of this three step algorithm was compared with a modified gold standard that combined tissue culture cytotoxicity (CYT) and DPCR. A separate investigation was performed to evaluate the sensitivity of the GDH-Q as a screening test, and toxigenic CD was found in 1.9% of 211 GDH-Q-negative specimens. The overall sensitivity, specificity, and positive and negative predictive values, respectively, were for GDH-Q/AB-Q/DPCR: 83.8\%, 99.7\%, 97.1\% and 97.9\%; and for CYT alone: 58.8\%, 100\%, 100\%, and 94.9\%. In comparison, the sensitivity and specificity of DPCR were estimated to be 97.5\% and 99.7\%, respectively, using the same modified gold standard. Neither CYT nor toxin EIA was sufficiently sensitive to exclude toxigenic CD, and combining EIAs with CYT in a three-step algorithm failed to substantially improve sensitivity. DPCR is a sensitive and specific method for the detection of toxigenic CD that can provide same day results at a cost-per-positive test comparable to other methods. A three-step algorithm in which DPCR is used to analyze GDH EIA-positive, toxin EIA-negative specimens provides a convenient and specific alternative with rapid results for 87.7\% of specimens, although this approach is less sensitive than performing DPCR on all specimens.
INTRODUCTION

*Clostridium difficile* (CD) is the leading cause of infectious diarrhea in hospitalized patients. New challenges have been posed by the emergence of highly virulent CD strains that may be refractory to standard treatment and cause disease even in immunocompetent individuals without prior antibiotic exposure (11, 28). Rapid and accurate laboratory diagnosis is critical to reduce the morbidity from *C. difficile*-infection (CDI) and allow the implementation of specific infection control measures.

Methods for the detection of toxigenic CD have long been unsatisfactory. The tissue-culture assay for cytotoxin B (CYT) is often considered the “gold standard” for diagnosis (10, 29), but many reports have documented the failure of CYT to detect symptomatic and even life-threatening cases of CDI (14, 33, 44), and the poor sensitivity of CYT in comparison to toxigenic culture (18, 22, 39). The technical complexity of CYT, as well as a requirement for 24-48 hours of incubation, has resulted in the widespread replacement of CYT with toxin immunoassays that provide results within minutes. In a 2008 College of American Pathologists report of proficiency results, 95% of laboratories reported using an EIA kit to detect CD toxin (6). CD immunoassays are often adopted as stand-alone assays based on validation against CYT, but studies that have employed more sensitive gold standards have documented that rapid toxin EIAs have unacceptably low sensitivities ranging from 32-79% (3, 20, 23, 44, 50).

Toxigenic culture, when performed under optimal culture conditions and combined with a sensitive and specific toxin detection method, is regarded as the most sensitive method of toxigenic CD detection (18, 44, 50), but complexity and a prolonged turnaround time have discouraged its routine use. An alternative approach has been to test for the glutamate dehydrogenase antigen (GDH) antigen of CD as a surrogate for culture. GDH EIAs have been reported to be highly sensitive for CD detection, allowing same day reporting of negative results, but positive results must be followed by a sensitive and specific test to differentiate between toxigenic and non-toxigenic
strains (4, 57, 58). In this study, we used the Wampole C DIFF QUIK CHEK EIA (GDH-Q), a rapid GDH assay that requires less technical time and expertise in comparison to microtiter plate immunoassays, followed by the TOX A/B QUIK CHEK (AB-Q) on GDH-Q-negative specimens. The choices for a second-step assay to detect toxigenic CD in GDH-positive specimens have included toxigenic culture, CYT or toxin EIAs (20, 23, 46, 57), but the insensitivity of CYT and toxin EIAs potentially leaves many cases of CDI undetected.

An alternative, highly sensitive method to detect toxigenic *C. difficile* is real-time PCR (12, 44, 54, 60), with sensitivity values ranging from 83.6% to 93.4% and specificity from 93.9% to 98.2%, respectively, when compared to toxigenic culture (50, 54, 60). Real-time PCR can be completed on the day of specimen submission, thus providing same day results. However, PCR techniques have not been not widely used for stool specimens, due primarily to budgetary issues, as well as the challenge of extracting nucleic acids from feces and separating template DNA from potentially interfering substances.

We have developed a sensitive and specific real-time PCR assay (DPCR) for the CD toxin B-encoding *tcdB* gene, which can be performed directly on stool specimens. Nucleic acids can be efficiently extracted from feces using the NucliSENS miniMAG system, a semi-automated magnetic silica bead extraction system to remove inhibitors and purify nucleic acids. Results were compared with the total yield of positive specimens detected by CYT and DPCR. Our observations indicate that a multi-step algorithm consisting of GDH EIA, toxin EIA and selective DPCR can provide a specific and cost-effective approach to the laboratory detection of toxigenic CD that is more sensitive than CYT or EIA alone, but not as sensitive as toxigenic culture or DPCR.
MATERIALS & METHODS

Study description. From January 2008 through July 2008, 699 soft or liquid stool samples from adult patients at Harborview Medical Center were submitted for detection of toxigenic CD by both the tissue culture cytotoxicity assay (CYT) and a three-step EIA/PCR algorithm. Specimens were refrigerated upon receipt and duplicates from the same day were excluded. The *C. difficile* EIA/PCR algorithm began with the C DIFF QUIK CHECK (GDH-Q) EIA, with a positive GDH-Q result triggering the second-step TOX A/B QUIK CHEK (AB-Q) (Wampole/TechLab, Blacksburg, VA) EIA for toxins A and B. Specimens with indeterminate EIA results (GDH-Q-positive, AB-Q-negative) were tested by direct stool PCR (DPCR) to detect the *tcdB* gene. AB-Q-positive specimens were considered positive and GDH-Q-negative specimens considered negative for toxigenic CD, and were not tested further by PCR in view of previous reports documenting the high specificity and sensitivity of the QUIK CHEK toxin and GDH EIAs, respectively (20, 23, 47).

EIAs. The QUIK CHEK assays are lateral flow, membrane-bound enzyme immunoassays that are visually interpreted. The C DIFF QUIK CHECK (GDH-Q) detects the glutamate dehydrogenase antigen of CD, whereas the TOX A/B QUIK CHEK (AB-Q) detects toxins A and B without differentiation of the toxins. Each EIA was contained in a separate cassette. Both assays were performed per manufacturer’s instructions.

CYT. Specimens submitted for CD cytotoxin detection by tissue culture assay were processed within 24 hours by the University of Washington Clinical Virology Laboratory. After dilution and centrifugation of fecal material in Hanks’ solution, the supernatant was filtered and added to human diploid fibroblast cell monolayers with and without CD antitoxin (TechLab, Blacksburg, VA). Samples producing characteristic cytopathic effects only in the absence of CD antitoxin were considered positive for CD toxin B, with the final interpretation of results after 48 hrs of incubation.
**DPCR extraction.** Non-liquid stools were diluted and mixed well with sufficient molecular grade phosphate buffered saline (Invitrogen, Carlsbad, CA) to allow aspiration with a wide bore pipette. The liquefied specimen was thoroughly mixed by vortexing and then centrifuged for 1 min at low speed (80 x g) to sediment solid fecal material while retaining bacteria in the supernatant fluid. An occasional mucoid specimen required further mixing and centrifugation at 320 x g, with careful aspiration of supernatant. A 100 µl aliquot of supernatant fluid aspirated from just above the sediment with a genomic wide bore tip was lysed with NucliSENS Lysis Buffer, and nucleic acids extracted and eluted with the NucliSENS miniMAG system (bioMérieux, Durham, NC) per manufacturer’s instructions. The final eluted specimen volume was 60 µl.

**DPCR real-time PCR.** Amplification to detect tcdB was performed on the Rotor-Gene-Q 6000 thermocycler (QIAGEN Inc., Valencia, CA). The NK104/NK105 primer set, designed by Kato et al. (27), was used to amplify a 204 bp sequence. Each 20 µl DPCR reaction contained 10 µl of 2X QuantiTect SYBR Green Master Mix (QIAGEN, Valencia, CA), 1 µl each of 10 µM forward and reverse primers, 6 µl of H2O, and 2 µl of extracted specimen. Amplification began with a 15 min step at 95°C, followed by 40 cycles of 15 s at 95°C, 25 s at 53°C, and 20 s at 72°C, finally followed by a 30 s step at 72°C before a stepwise (1°C/5 s) temperature increase to 90°C. Detection of the 16S rRNA-encoding gene of CD was also performed in a separate reaction on each extracted GDH-Q positive specimen as an internal control, using primer set CD3/CD6 (45) (<5'-'GGCGGCGTGCCTAAC-3'> and <5'-'TGGCTCACCTTTGATATTC-3'>) and the same amplification conditions as for tcdB detection. Distinct single melt peaks at 76.3°C ± 1°C and 82.0°C ± 1°C were considered positive for tcdB and CD 16S rRNA gene detection, respectively. Because several GDH-Q-positive specimens had no detectable CD 16S rRNA DNA and were culture-negative for CD, an additional amplification control of heat-extracted DNA from a nontoxigenic CD isolate was added to each extracted specimen and a reagent control in a 1/15 dilution. Subsequently, amplification with universal
bacterial 16S rRNA gene primers (25) was employed as an internal extraction and amplification control, with an expected melt peak of 86.5 ± 2°C and cycle time of less than 20.
RESULTS

In data not shown (32), the detection of toxigenic CD by DPCR performed as a second step assay on Triage C difficile Panel indeterminate (GDH-positive, toxin A-negative) specimens was validated by comparing detection with the combined yield of positives by the cytotoxin assay (CYT) and a toxigenic culture method that identified toxigenic CD colonies by real-time PCR for \textit{tcdB}. The Triage EIA/DPCR combination detected 93.4% (71/76) of specimens positive for toxigenic CD, with specificity, PPV, and NPV values of 100%, 100% and 98.9%, respectively. The Triage panel is a membrane bound lateral flow EIA that is no longer commercially available.

For evaluation of the three-step GDH-Q/AB-Q/DPCR, all DPCR and CYT positives were considered true-positives (Table 1), based on the DPCR performance described above and the high specificity of CYT. All CYT-positive specimens were also positive by GDH-Q. CYT was negative on two of 28 specimens that were positive by both GDH-Q and AB-Q.

In the initial evaluation of data, 585 (83.7% of 699) fecal specimens that were negative by GDH-Q and CYT assays were considered to be true negatives (Table 1). However, in December 2008, DPCR performed on 211 consecutive GDH-Q-negative specimens revealed that 1.9% (n=4) of GDH-Q-negative specimens contained toxigenic CD, which was confirmed by toxigenic culture, including GDH-Q, AB-Q and DPCR testing of CD isolates. By applying this false-negative rate to the 585 GDH-Q-negative specimens, 11 additional specimens were designated as true-positives, with 574 remaining as true-negatives, as shown in Table 2.

After adjusting for false negative results, GDH-Q was 86.3% (69/80) sensitive with a 60.5% positive predictive value (Table 3) but only 92.7% specificity. AB-Q performed on GDH-Q-positives was only 32.5% sensitive but highly specific (99.7%). CYT achieved only 58.8% (47/80) sensitivity, whereas the three-step...
algorithm of GDH-Q, AB-Q, and DPCR was more sensitive, detecting 83.8% (67/80) of positives, even though two GDH-Q-positive, CYT-positive specimens were not detected by DPCR. The difference in detection of positives between CYT and the EIA/DPCR combination was significant (p< 0.001 by McNemar’s test for paired proportions) and represented a 43% increase in the yield of positives over CYT alone. An algorithm combining the GDH-Q and AB-Q EIAs with CYT testing of GDH-Q-positive/AB-Q-negative specimens would only have improved sensitivity to 63.8% (51/80). In additional data not shown, the presence of toxigenic CD was confirmed in ten out of ten consecutive CYT-negative, DPCR-positive specimens by anaerobic culture followed by GDH-Q, AB-Q, and DPCR performed on suspensions of CD isolates.

**Repeat specimens after GDH-Q-negative results.** We investigated the value of submitting additional specimens after an initial negative GDH-Q result on patients with no history of a positive toxin result. From January through July 2008, repeat GDH-Q tests were performed for 171 patients on 194 specimens submitted one to seven days after the initial test. Duplicate specimens from the same day were rejected. All specimens submitted one to three days after the first test were either GDH-Q-negative (117/119) or GDH-Q-positive/AB-Q-negative/DPCR-negative (2/119). Of 75 repeat specimens tested at four to six days, thirteen were GDH-Q-positive, with six (8.0% of 75) of thirteen positive for toxigenic CD by either AB-Q (n = 3) or DPCR (n=3). The difference in yield of toxin-positive samples between the two collection periods was significant (p< 0.02) by chi-square contingency table with Yates correction.

**Cost estimates.** Table 3 displays the estimated costs of the various approaches to detect toxigenic CD. Figures were derived from our 2007-2008 test volume, estimated labor and benefit costs of $50/hour (U.S. dollars), and the yield of toxigenic CD positives shown in Tables 1 and 2. Labor and materials costs for quality control, test preparation, cleanup, and result interpretation were included, but equipment...
expenses were excluded. Note that costs may vary significantly according to region and specimen volume.

Estimates of annual laboratory expenses for toxigenic CD detection ranged from $47,000 and $56,000 for AB-Q alone or a two-stage GDH-Q/AB-Q algorithm, respectively, to $126,000 for DPCR as either a stand-alone test or in the 3-step EIA/DPCR protocol (Table 3). However, the two-step GDH-Q/AB-Q approach would miss 231 (66.7%) of 346 positive specimens detectable by a stand-alone DPCR test, and assuming that all AB-Q positives are also GDH-Q positive, AB-Q yield of positives would be equivalent to that of the two-step GDH-Q/AB-Q. A three-step EIA/CYT protocol would miss 138 (39.9%) of 346, with 505 (16.3% of 3100) specimens requiring 24 to 48 hours of incubation before reporting, whereas the three-step EIA/DPCR algorithm would miss only 49 (14.2%) positives, and processing could be completed within one day.

EIA costs per specimen were lowest at $15 for AB-Q and $18 for the two-step GDH-Q/AB-Q, respectively, but when evaluated on a cost-per-positive test basis, the PCR-based three-step algorithm was comparable to EIA testing (Table 3). A less expensive approach that employs DPCR is a two-step GDH-Q/DPCR algorithm, which would delay results for 4% of positives that would otherwise be detected by the AB-Q in 30 to 40 minutes. DPCR performed on all specimens had the lowest cost per positive specimen and highest sensitivity (346 out of 354 positives), but the three-step EIA/DPCR option has the advantage of shorter turnaround time and ease of test performance for the 87.7% of specimens that do not require DPCR.

**Statistical analysis.** Graphpad software ([http://graphpad.com/quickcalcs](http://graphpad.com/quickcalcs)) was used to determine 95% confidence intervals and significance by McNemar’s test of proportions and the chi-square test with Yates correction.
DISCUSSION

The accurate diagnosis of CD infection (CDI) has become increasingly important, yet the laboratory methods for diagnosis have remained problematic and controversial. The tissue culture assay for *C. difficile* cytotoxin (CYT) is often cited as the “gold standard” for toxigenic CD detection (10, 29). However, while the CYT is highly specific, it is only moderately sensitive and has been well documented to miss cases of CDI (14, 33, 35, 44). For this reason, the Society for Healthcare Epidemiology of America (SHEA) has recommended that both stool culture and toxin testing be performed, “preferably with confirmation of organism toxicity if a direct stool toxin test is negative or not done” (21). Nevertheless, this recommendation is not followed by the vast majority of clinical laboratories, which largely rely on insensitive toxin EIA kits (6).

Proposed multi-step diagnostic algorithms have improved efficiency but continue to depend upon insensitive combinations of EIA and CYT assays (23, 57). The goal of the present study was to determine whether DPCR could be integrated with EIAs to optimize the detection of toxigenic CD while taking advantage of the convenience of the rapid commercial assays. As suggested by previous investigators (9, 51, 58), our study used a GDH screening method to rapidly report the majority of specimens. The GDH-Q, a lateral flow EIA which can be completed in 30 to 40 minutes, allowed 84% of specimens to be reported as negative for CD. Second-step toxin EIA testing by the AB-Q, also a 30 to 40 minute assay, identified 4% of specimens as toxin positive, leaving 12% for DPCR testing (Figure 1).

Consistent with SHEA recommendations, our DPCR method had been validated using a gold standard of both toxigenic culture (with real-time PCR detection of *tcdB* in colonies) and CYT. Against this standard, we found DPCR to be a sensitive and specific method for the detection of toxigenic CD in fecal specimens (32) and, therefore, did not include toxigenic culture in this study but instead compared results with the total yield of positives by CYT and DPCR, as shown in Table 1.
A shortcoming of this approach was the assumption of the high sensitivity and negative predictive value of GDH-Q. Although Reyes et al. reported that the GDH-Q detected 46 of 46 specimens with toxigenic CD (47), and data from Gilligan suggest a high negative predictive value (23), we found that four (1.9%) of 211 consecutive GDH-Q negative specimens in a subsequent time period contained the \textit{tcdB} gene by DPCR testing, which was confirmed by toxigenic culture. Chart review of the patients with falsely negative GDH-Q results revealed that three of four received antibiotic treatment for CDI based on the positive DPCR results. If DPCR had not been performed on the GDH-Q-negative specimens, all of which were also CYT-negative, the sensitivity and NPV of the three-step QUICK CHEK EIA/DPCR algorithm would have appeared to be 97.1\% and 99.7\%, whereas the more accurate adjusted values were determined to be 83.8\% and 97.9\%, respectively (Figure 1, Table 2).

It should be noted that our laboratory performs PCR and toxigenic culture on request when warranted by the clinical presentation, regardless of the GDH-Q results. For example, on one occasion subsequent to this investigation, we encountered a patient with refractory diarrhea, colitis by endoscopy and repeated specimens negative by GDH-Q and a toxin immunoassay. After detection of toxigenic CD by both DPCR and toxigenic culture, the patient underwent appropriate treatment for CDI. Occasional specimens may contain inhibitory substances or insufficient numbers of CD organisms to produce a positive EIA result. In such cases, DPCR and toxigenic culture can be very helpful and should be employed irrespective of a negative EIA result when clinical suspicion of CDI is high.

The results of our EIA/DPCR algorithm are similar to the results of direct real-time PCR assays described by Sloan et al. (50) and Stamper et al. (54), who reported sensitivities of 86\% and 83.6\%, and specificities of 97\% and 98.2\%, respectively, in their comparisons with toxigenic culture results. The sensitivity of our three-step
approach was less than that of the method of Peterson et al., who found their real-time PCR to be 93.4% sensitive and 97.4% specific when compared to a gold standard that combined clinical information (≥3 loose stools/day) with ≥2 positive toxin assays (44). To estimate the sensitivity and specificity of performing DPCR on all specimens, 26 specimens that were positive by GDH-Q, AB-Q and CYT-Q but not tested by DPCR (per the three-step algorithm) must be assumed to be DPCR-positive. Using this assumption, the sensitivity and specificity of DPCR as a stand-alone test are estimated to be 97.5% (78/80) and 99.7% (617/619), respectively.

For the real-time PCR detection of toxigenic *C. difficile*, we chose to target a conserved region of the *tcdB* gene (24), in accordance with most PCR-based CD assays (12, 24, 44, 60). The *tcdB* gene is a preferable target to *tcdA* because it allows the detection of increasingly prevalent toxin A-negative toxin B-positive strains (4, 13, 15, 26, 27, 30, 36, 41, 48, 49), as well as strains producing both toxins A and B. Strains that carry *tcdB* but exhibit low levels of expression (38) or produce toxin A only (17) appear to be very uncommon. Although Sloan et al. have suggested *tcdC* to be a suitable target for PCR detection of toxigenic *C. difficile* (50), the variability of *tcdC* (53, 55) and its dispensability for virulence raise concerns about its long-term stability as a diagnostic target. A recent study has documented that toxin B is essential for CD virulence (37) but five of the strains described by Sloan as toxigenic contained only the *tcdA* and *tcdC* genes and may actually have been non-toxigenic due to the absence of toxin B.

In our study, CYT detected only 58.8% of positives, comparable to previous studies describing the insensitivity of CYT (18, 22, 39, 46). The AB-Q, performed on GDH-Q-positive specimens, was more than 99% specific but very insensitive (32.5%), consistent with previous studies of the AB-Q (23, 47) and other toxin immunoassays (44, 50). High specificity (99.7%), rapid turnaround time and technical simplicity make the AB-Q a useful intermediate-step assay. However, the failure to detect toxin in 41 of 67 specimens found to be DPCR-positive highlights the unreliability of negative toxin EIA results. Two specimens with positive AB-Q results on GDH-
positive specimens were not confirmed by CYT and were unavailable for DPCR testing. Gilligan has also noted a small percentage of AB-Q positives that could not be confirmed by CYT (23). These may have been cross-reacting C. sordellii isolates, as mentioned in the AB-Q package insert. We have observed positive AB-Q results with the C sordellii ATCC 9714 strain and one patient C sordellii isolate, both of which were GDH-Q-negative. Restricting AB-Q testing to GDH-Q-positive specimens thus might reduce the likelihood of false-positive results.

Submission of repeat specimens from patients with an initial GDH-Q-negative specimen and no history of a previous positive toxin result produced positive toxin results in 3.0% of specimens tested within 7 days of the negative initial test, comparable to previous reports of 1.3-2.1% (1, 16, 40). All repeat GDH-Q specimens submitted within one to three days of the first test were negative, whereas 8% of those specimens submitted after four to six days were found to be toxin positive. This is similar to the results of Cardona and Rand, who recommended against repeating AB-Q testing within 48 hours of a negative result (16).

Clearly, GDH-Q is insufficiently specific, and CYT and AB-Q are insufficiently sensitive to be relied upon as assays for toxigenic CD, whether used alone or in combination with each other, although EIA testing is the least expensive method for the laboratory budget. The incorporation of DPCR into an algorithm using EIAs as screening steps increases overall costs of testing but not the cost per case detected (Table 3). The increased number of cases detected (approximately 182 specimens per year positive by three-step EIA/DPCR but negative by EIA) may compensate for higher labor and reagent costs by reducing the nosocomial spread of CDI. Infected patients are an important reservoir of CD in institutional settings, and person-to-person transmission has been demonstrated in 10 to 20% of cases of hospital-associated CDI (8, 56). Emerging 027/NAP1 epidemic strains may have even greater transmissibility due to enhanced toxin and spore production (2).
Patients who develop nosocomial CDI incur increased attributable costs ranging from $3,791 to $6,959 per episode (19, 34, 42, 52) (median costs adjusted to 2009 Consumer Price Index dollars (59)) and are hospitalized from 2.9 to 5.5 additional days (31, 34, 42, 52), compared to patients without CDI. The additional annual costs of a DPCR or three-step EIA/DPCR algorithm are justified by the earlier detection of CDI, which would reduce the number of cases resulting from nosocomial transmission. In comparison to the toxin EIA (AB-Q) alone, the three-step EIA/DPCR algorithm would cost $437 per additional case of CDI detected, while a strategy of performing DPCR on every specimen would cost $343 per additional case detected, as calculated from Table 3 figures. The additional expense is even more modest when compared to the “gold standard” of tissue culture cytotoxin assay (CYT), with the three-step EIA/DPCR algorithm costing $175 per additional case detected and DPCR only $110 per additional case detected. A further justification for improving the sensitivity of CD detection is the resulting decrease in length of stay due to earlier diagnosis. With the daily cost of hospitalization attributable to CDI estimated at between $1,300 and $2,000 (31, 34, 42, 52), implementing DPCR or a three-step EIA/DPCR algorithm could save more than $200,000 annually simply by making the diagnosis of CD one day earlier in cases missed by EIA, as these patients would otherwise be likely to require repeat testing.

The choice of real-time PCR methods for direct detection from stools now includes the Xpert C. difficile Test (Cepheid, Sunnyvale, CA), the ProGastro Cd assay (Prodesse, Waukesha, WI) and the BD GeneOhm Cd assay (BD Diagnostics, San Diego, CA), with published GeneOhm sensitivity values of 83.6 and 93.9%, and NPVs of 97.1% to 99.2%, when compared to toxigenic culture (7, 54), although unresolved GeneOhm results were as high as 7.3% in one study (7). Our observations confirm that DPCR is a sensitive, specific and cost-effective method for the detection of toxigenic CD. A multi-step algorithm consisting of a GDH EIA screen followed by a specific toxin EIA screen and direct real-time PCR provides a convenient, rapid and specific alternative strategy, but with the trade-off of some reduction in sensitivity.
ACKNOWLEDGMENTS

The NucliSENS miniMAG instrument and reagents were provided by bioMérieux, Inc. for the duration of the 2005 study. The authors are grateful to Carol I. Johnson, Cheryl A. McMillan, Thomas R. Smith, and Audrey A. Twite for their professionalism and technical support.
REFERENCES


difficile detection increases diagnostic yield. 105th General Meeting of the American Society for Microbiology, abstract C-335.


Stool specimens

Step 1
QUIK CHEK
GDH

Step 2
QUIK CHEK
Toxin A/B

Step 3
Direct PCR (DPCR)

Toxigenic C. difficile not detected (82.1%); false negatives (1.6%)

Toxigenic C. difficile (3.7%); presumptive false positives (0.3%)

Toxigenic C. difficile (5.9%)

Toxigenic C. difficile not detected (6.2%); presumptive false negatives (0.3%)
TABLE 1. Performance of C DIFF QUIK CHEK EIA, TOX A/B QUIK CHEK EIA, cytotoxin assay and direct PCR for detection of toxigenic *C. difficile*

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>GDH-Q</th>
<th>A/B-Q</th>
<th>CYT</th>
<th>DPCR</th>
<th>Interpretation of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>585</td>
<td>-</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: GDH-Q, C DIFF QUIK CHEK EIA; AB-Q, TOX A/B QUIK CHEK EIA; CYT, cytotoxin tissue culture assay; DPCR, direct real-time PCR detection of *tcdB*; ND, not done.

<sup>b</sup>PCR results: 1.9% (4/211) of consecutive GDH-Q-negative specimens in a subsequent time period were DPCR-positive for *tcdB*; therefore, 11 of 585 are expected to be GDH-Q-negative, DPCR-positive as shown in Table 2.
TABLE 2. Detection of toxigenic *C. difficile* by cytotoxin, GDH-Q\(^a\) EIA, and sequential algorithms\(^b\) compared with gold standard (cytotoxin assay and PCR)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>No. of specimens</th>
<th>Comparison to cytotoxin and direct PCR(^c) results</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>% PPV (95% CI)</th>
<th>% NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxin (tissue culture)</td>
<td>Positive</td>
<td>47 0</td>
<td>Either positive (^d)</td>
<td>58.8 (47.8-68.9)</td>
<td>100 (94.9-100)</td>
<td>100 (94.9-100)</td>
<td>100 (94.9-100)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>33 619</td>
<td>Negative</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>GDH-Q(^a)</td>
<td>Positive</td>
<td>69 45</td>
<td>% Sensitivity (95% CI)</td>
<td>86.3 (76.9-92.3)</td>
<td>92.7 (90.4-94.5)</td>
<td>60.5 (51.3-69.0)</td>
<td>98.1 (96.6-99.0)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>11 574</td>
<td>% Sensitivity (95% CI)</td>
<td>(23.2-43.4)</td>
<td>(98.8-100)</td>
<td>(76.3-99.1)</td>
<td>(89.6-93.8)</td>
</tr>
<tr>
<td>Two-step GDH-Q→AB-Q(^a)</td>
<td>Positive</td>
<td>26 2(^d)</td>
<td>% Sensitivity (95% CI)</td>
<td>32.5 (23.2-43.4)</td>
<td>99.7 (98.8-100)</td>
<td>92.9 (76.3-99.1)</td>
<td>92.0 (89.6-93.8)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>54 617</td>
<td>% Sensitivity (95% CI)</td>
<td>(74.0-90.4)</td>
<td>(98.8-100)</td>
<td>(89.4-99.8)</td>
<td>(96.5-98.8)</td>
</tr>
<tr>
<td>Three-step GDH-Q→AB-Q→DPCR(^a)</td>
<td>Positive</td>
<td>67 2(^d)</td>
<td>% Sensitivity (95% CI)</td>
<td>83.8 (74.0-90.4)</td>
<td>99.7 (98.8-100)</td>
<td>97.1 (89.4-99.8)</td>
<td>97.9 (96.5-98.8)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>13 617</td>
<td>% Sensitivity (95% CI)</td>
<td>(76.9-92.3)</td>
<td>(90.4-94.5)</td>
<td>(51.3-69.0)</td>
<td>(96.6-99.0)</td>
</tr>
</tbody>
</table>

\(^a\)Abbreviations: AB-Q, TOX A/B QUIK CHEK EIA; GDH-Q, C DIFF QUIK CHEK EIA; DPCR, direct real-time PCR; CI, confidence interval.

\(^b\)GDH-Q was performed on all specimens, with AB-Q EIA performed on GDH-Q-positives, and DPCR on indeterminate (GDH-Q-positive, AB-Q-negative) specimens.
GDH-Q-negative specimens were not tested by DPCR in this study, so the rate of DPCR positivity (1.9%) in 211 GDH-Q-negative, CYT-negative specimens from a subsequent time period was applied to 585 GDH-Q-negatives, resulting in 11 specimens designated as GDH-Q negative, DPCR-positive.

*Cytotoxin-negative, DPCR not done.
<table>
<thead>
<tr>
<th>Assay</th>
<th>No. specimens positive for toxigenic CD</th>
<th>Annual Cost (US$)</th>
<th>Cost (US$)/specimen</th>
<th>Cost (US$)/positive specimen</th>
<th>Daily Processing time</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB-Q</td>
<td>115</td>
<td>46,562</td>
<td>15.02</td>
<td>405</td>
<td>&lt;1 h</td>
</tr>
<tr>
<td>Two-step GDH-Q→AB-Q</td>
<td>115</td>
<td>55,942</td>
<td>18.05</td>
<td>486</td>
<td>&lt;1.5 h</td>
</tr>
<tr>
<td>CYT</td>
<td>208</td>
<td>110,546</td>
<td>35.66</td>
<td>533</td>
<td>≤48 h</td>
</tr>
<tr>
<td>Three-step GDH-Q→AB-Q →CYT</td>
<td>208</td>
<td>75,680</td>
<td>24.41</td>
<td>365</td>
<td>&lt;1 h (87.7%)</td>
</tr>
<tr>
<td>DPCR</td>
<td>346</td>
<td>125,767</td>
<td>40.57</td>
<td>363</td>
<td>≤5 h</td>
</tr>
<tr>
<td>Two-step GDH-Q→DPCR</td>
<td>297</td>
<td>109,193</td>
<td>35.22</td>
<td>368</td>
<td>&lt;1 h (83.7%)</td>
</tr>
<tr>
<td>Three-step GDH-Q→AB-Q →DPCR</td>
<td>297</td>
<td>126,121</td>
<td>40.68</td>
<td>425</td>
<td>≤5 h (12.3%)</td>
</tr>
</tbody>
</table>
Estimated cost per test, with labor and benefits at $50/h:  AB-Q $15.02 batched, $21.68 singly; GDH-Q $14.51 batched; CYT $14.51 batched;  DPCR $40.57 in batch of nine tests, $184 singly, $127 in avg batch of 1.5 tests.

Based on annual volume of 3100 specimens (506 tested in 2nd step, 381 in 3rd step), 11.4% (n=354) prevalence, and data from Tables 1 and 2.

For abbreviations, see Table 1, footnote a.

Yield assumes all GDH-Q-negative specimens to be AB-Q-negative.

Yield assumes all GDH-Q-positive, AB-Q-positive specimens to be DPCR-positive.