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# Evaluation of BD GeneOhm CDiff PCR Assay for Diagnosis of Toxigenic *Clostridium difficile* Infection

Elizabeth Kvach

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**EVALUATION OF BD GENE OHM CDIFF PCR ASSAY FOR DIAGNOSIS OF  
TOXIGENIC *CLOSTRIDIUM DIFFICILE* INFECTION**

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements for the  
Degree of Doctor of Medicine\*

by

Elizabeth J. Kvach

2010

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## ABSTRACT

*Clostridium difficile* is the most common infectious cause of nosocomial diarrhea, affecting thousands of patients annually and exacting enormous costs on the U.S. health care system. Early diagnosis is critical to prevent transmission and reduce morbidity and mortality, yet sensitive and specific diagnostic tests with a quick turnaround time are lacking. The objective of this study was to determine if a new commercially available real time polymerase chain reaction (PCR) test would prove more rapid, sensitive and specific than standard methods for the diagnosis of *C. difficile* infection (CDI). BD GeneOhm™ Cdiff assay, a real-time PCR assay for detection of *C. difficile* toxin B (*tcdB*) gene, was compared with Tox A/B II™ ELISA and a two-step algorithm which includes C. Diff Chek-60™ Glutamate Dehydrogenase (GDH)-antigen assay followed by cytotoxin neutralization. Four-hundred liquid or semisolid stools submitted for diagnostic *C. difficile* testing were selected: 200 GDH antigen-positive and 200 GDH antigen-negative. All samples were tested by the C. Diff Chek-60™ GDH antigen, cytotoxin neutralization, Toxin A/B II™ ELISA, and BD GeneOhm™ Cdiff assay. Discrepant specimens were tested by toxigenic culture as an independent gold standard. Chart review was performed on patients with discrepant specimens. As BD GeneOhm™ Cdiff assay was not FDA-cleared at the time of study, PCR results were not clinically reported. Of 200 GDH-positive samples, 71 were positive by Tox A/B II, 88 were positive by the two-step method, 93 were positive by PCR, and 96 were positive by GDH-antigen only. Of 200 GDH-negative samples, 3 were positive by PCR only. Toxigenic culture was performed on 41 samples with discrepant results and 39 were culture-positive. After

culture resolution of discrepant, Tox A/B II detected 70 (66.7%), the two-step method detected 87 (82.9%), and PCR detected 96 (91.4%) of 105 true positives. The BD GeneOhm™ Cdiff assay was more sensitive in detecting toxigenic *C. difficile* than Tox A/B II ( $p < 0.0001$ ); however, the difference between PCR and the two-step method was not significant ( $p = 0.1237$ ). The BD GeneOhm™ Cdiff assay took a similar amount of time to perform as the Tox A/B II and was more rapid than the two-step method. Chart review revealed that 18 patients with cytotoxin-negative, PCR-positive discrepant samples were given 1-2 days of therapy ( $n = 8$ ), or no treatment at all ( $n = 10$ ). Yet symptoms resolved and no further *C. difficile* testing was requested for 13 of 18 patients for 6-8 months after hospital discharge. Only one patient had a subsequent cytotoxin positive stool submitted 22 days after the study sample was tested. Enhanced sensitivity and rapid turnaround time make the BD GeneOhm™ Cdiff assay an important advance in the diagnosis of toxigenic *C. difficile* infection. The BD GeneOhm™ Cdiff assay is significantly more sensitive than a commonly-used ELISA toxin assay and has a sensitivity and specificity comparable to the two-step method. Its turnaround time is similar to ELISA toxin assays and more rapid than the two-step method. Disadvantages to implementation of BD GeneOhm™ Cdiff assay include increased cost and potential treatment of asymptomatic carriers and mild, self-resolving disease.

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**ABBREVIATIONS**

|               |  |
|---------------|--|
| <b>BD</b>     | Becton Dickinson                       |
| <b>CCFA</b>   | Cycloserine-Cefoxitin-Fructose Agar    |
| <b>CDI</b>    | <i>Clostridium difficile</i> Infection |
| <b>CNA</b>    | Cytotoxin Neutralization Assay         |
| <b>EIA</b>    | Enzyme Immunoassay                     |
| <b>ELISA</b>  | Enzyme-Linked Immunosorbent Assay      |
| <b>FDA</b>    | Food and Drug Administration           |
| <b>GDH</b>    | Glutamate Dehydrogenase                |
| <b>MIC</b>    | Minimum Inhibitory Concentration       |
| <b>NPV</b>    | Negative Predictive Value              |
| <b>PCR</b>    | Polymerase Chain Reaction              |
| <b>PPV</b>    | Positive Predictive Value              |
| <b>RT-PCR</b> | Real-Time Polymerase Chain Reaction    |
| <b>Tcd</b>    | <i>Clostridium difficile</i> Toxin     |
| <b>YNHH</b>   | Yale-New Haven Hospital                |

## **INTRODUCTION AND BACKGROUND**

*Clostridium difficile* infection (CDI) is increasing as the most common infectious cause of nosocomial diarrhea in hospitalized patients in the United States (1). *C. difficile* infection (CDI) accounts for 15-25% of all cases of antibiotic-associated diarrhea (2). It is estimated that approximately half a million cases of CDI occur in the United States per year and 15,000-20,000 of these patients die from CDI (1). In the U.S., CDI is responsible for more deaths than all other intestinal infections combined (3). One study estimated that CDI costs an average of \$3600 more per patient with length of stay in the hospital extended by 3.6 days. When extrapolated, the cost of CDI disease burden to the United States health care system was \$1.1 billion annually (4). In response to this disease burden, hospitals have had to institute systems of early identification and isolation of *C. difficile*-positive patients in order to minimize morbidity and prevent spread to other patients (5). The effectiveness of early detection depends upon the speed and accuracy of the laboratory tests, which until now have been suboptimal. Commercial polymerase chain reaction (PCR) tests are now becoming available, but their performance compared to current methods has not been established.

### **Microbiology of *Clostridium difficile***

*C. difficile* is an obligately anaerobic gram-positive rod. It forms spores that can remain in the environment for months and are resistant to the low pH of stomach acid (6, 7). Spores have been found to be resistant to non-chlorine based cleaning agents and heat of common hospital laundry cycles, even cross-contaminating bed linens during laundering (8). Other virulence factors include: adhesins, proteases, and toxin production (1).



## Pathogenesis

Typically *C. difficile* disease is caused by administration of broad-spectrum antibiotics to which *C. difficile* is not susceptible, leading to disruption of normal colonic bacterial flora and overgrowth of *C. difficile* after ingestion of spores. Spores germinate and vegetative forms multiply, after which they adhere to and penetrate the mucous layer coating colonic epithelium with the aid of proteases, including a hyaluronidase, and flagella. This then allows the bacteria to adhere to enterocytes via multiple adhesins and complete the colonization phase (7, 9).

After colonization, *C. difficile* enters the toxin production phase. Its main virulence factors are two protein exotoxins, toxin A (enterotoxin) and toxin B (cytotoxin), which are the largest bacterial single-molecule exotoxins known (6). The toxin A (TcdA) and toxin B (TcdB) genes are located within a pathogenicity locus that is comprised of five genes, including TcdC, TcdE, and TcdR which encode regulatory proteins (9).

Production of Toxin A and B is negatively regulated by the TcdC gene locus (10). Both toxins act similarly by being endocytosed into colonic cells where they disrupt the actin cytoskeleton and tight junctions, leading to decreased transepithelial resistance, fluid accumulation and ultimately death of the intestinal epithelium. Toxin B is about 1000 times more potent than toxin A, having 100-fold higher enzymatic activity per toxin molecule. Both toxins stimulate monocytes to produce  $\text{TNF}\alpha$  and IL-8, leading to extravasation and tissue infiltration by neutrophils, which in turn cause an inflammatory response and contribute to mucosal cell destruction and pseudomembrane formation (7, 11).

Toxins A and B are produced in the late log and stationary phases. Their levels are impacted by the availability of nutrients, temperature and sub-inhibitory levels of antibiotics (12). According to one in-vitro study, toxigenic *C. difficile* showed earlier and increased rates of toxin production when exposed to sub-MIC concentrations of vancomycin, metronidazole and linezolid, as compared to controls not exposed to antibiotics (13).

Toxin B is necessary to produce clinical disease, whereas toxin A alone is insufficient. TcdA-negative and TcdB-positive strains have been reported (1). Nontoxigenic strains of *C. difficile* lack TcdA and TcdB (14). The pathogenicity locus is replaced with a non-coding sequence (1). Patients can be asymptotically colonized with strains of *C. difficile*. There is some evidence to suggest that asymptomatic carriage is associated with higher levels of IgG against *C. difficile* toxins and that colonized patients with lower levels of immunoglobulin are more likely to develop symptomatic disease (4).

A relatively recent epidemiologic occurrence has been the emergence of a hypervirulent epidemic strain of *C. difficile* associated with hospital outbreaks in several countries with high rates of complications and mortality. The strain – labeled BI/NAP1/027 for short – is restriction endonuclease analysis group BI, pulse-field gel electrophoresis type NAP1, and polymerase chain reaction ribotype 027. Its unique virulence factors are characterized by increased levels of toxin A and B; synthesis of a third toxin, binary toxin; and resistance to fluoroquinolones. The first factor is thought to be largely due to deletion of the *tcdC* gene that is a repressor of toxin A and B production. These strains

produce up to 23-fold more toxin than other strains without the same mutation (9, 15, 16).

Another epidemiologic concern is the question of rising incidence of community-acquired CDI in which patients have no recent history of hospitalization or antibiotic exposure. Two surveys by the Centers for Disease Control in 2005-2006 found the rates of community-acquired CDI per 100,000 population in Philadelphia and Connecticut to be 7.6 and 6.9, respectively (17, 18). An earlier study in Boston found a rate of 7.7 per 100,000 person years with 35% of the study population not having received antibiotics in the six weeks prior to onset of symptoms (19). While the rate of CDI in the community is still much lower than in hospitalized patients, infections are increasingly being reported in populations that were otherwise thought to be at low-risk (1, 11).

### **Risk Factors for *C. difficile* Infection**

The major risk factors for CDI that have been identified are: broad-spectrum antibiotic exposure, specifically to clindamycin, cephalosporins, extended-spectrum beta lactams, and aminopenicillins; prolonged hospitalization (>72 hours); and older age (>65 years) (1, 2, 9). Other associated risk factors include concomitant use of antacids, immunosuppressive states or therapy (e.g. methotrexate), and inflammatory bowel disease (20). There is conflicting evidence about whether or not fluoroquinolones increase the risk of developing CDI, though BI/NAP1/027 strains have been documented to be resistant to this antibiotic class (21).

## Signs and Symptoms

The typical clinical presentation of CDI is the onset of watery diarrhea with a typical foul-smelling odor in a patient who has received broad-spectrum antibiotics and been hospitalized for greater than 72 hours. Fever and abdominal pain or cramping can also be present, though they may be absent. Laboratory data may show leukocytosis, presence of fecal leukocytes, and hypoalbuminemia. The presentation of CDI can range from mild diarrhea to fulminant colonic failure. Rarely, diarrhea will be absent, as in a patient with paralytic ileus, particularly in association with narcotics administration (2, 22).

While CDI should be the first entity ruled out in a patient with antibiotic-associated diarrhea who has been hospitalized >72 hours, the differential diagnosis includes: antibiotic side effects; failure of colonic flora to catabolize carbohydrates; and other infectious etiologies such as *Staphylococcus aureus* (including methicillin-resistant strains), enterotoxin-producing strains of *Clostridium perfringens*, *Salmonella* species, and *Klebsiella oxytoca*, though these are rare (23).

## Complications

Fulminant or severe complicated CDI includes the development of pseudomembranous colitis, which can lead to toxic megacolon or bowel perforation, septic shock and even death. This can necessitate intensive care unit admission and surgical intervention including colectomy. These complications are rare but when they do occur the costs of hospitalization are high (1).

## Treatment

For mild, uncomplicated disease, oral metronidazole for 10-14 days is the preferred first-line therapy, primarily because it is more cost-effective.<sup>1</sup> In severe or complicated cases, oral vancomycin is the recommended treatment (24). Intravenous metronidazole is reserved for patients who cannot take oral medications. One recent randomized, placebo-controlled, double-blind trial stratified by disease severity showed no statistically significant difference in outcomes for patients with mild CDI treated with oral metronidazole versus vancomycin. However, for severe disease, oral vancomycin was shown to achieve a significantly higher cure rate than metronidazole, perhaps secondary to the higher intra-colonic concentrations achieved as a result of poor systemic absorption (25). Both metronidazole and vancomycin have been associated with higher rates of colonization with vancomycin-resistant enterococci (VRE), though this has been insufficiently studied.

Alternative antimicrobial treatment options that have not been well studied include rifaximin PO, nitazoxanide PO, and ramoplanin. Adjunct treatments include toxin-binding resins (i.e. tolevamer and cholestyramine), intravenous immune globulin and probiotics, though evidence for success of these agents is mixed and unclear (20). A Cochrane review of the use of probiotics in *C. difficile* treatment found that in only one study had they been shown to reduce rates of disease recurrence, but otherwise there was no evidence to support their use as a sole treatment agent (26).

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<sup>1</sup> According to one estimate by Pepin, et al., a 10-day course of metronidazole costs approximately \$20 whereas a 10-day course of vancomycin is approximately \$600.

## Recurrence

Recurrence of CDI is likely due to the persistence of bacterial spores despite high intracolonic concentrations of antibiotic (assuming an oral form of antibiotic has been previously administered), which then germinate upon discontinuation of therapy.

However, up to half of recurrent cases have been found to be due to re-infection with a new strain (23). In spite of effective antibiotic therapy, up to 15-25% of patients will have recurrent disease after completion of treatment (27).

A recent meta-analysis assessing risk factors studied for recurrent CDI reported that only three factors were significantly associated: continued use of non-*C. difficile* antibiotics after diagnosis of CDI, concomitant receipt of antacid medications and older age (28).

However, these are also risk factors for acquisition of primary CDI as well. A more specific explanation of risk for recurrent CDI was proposed by Kyne and colleagues, who found that levels of both IgM and IgG against *C. difficile* toxin A were higher in patients with only a single episode of CDI than in patients who had experienced recurrent disease. They suggest that immune response to toxin A confers a protective effect against future infections, and that failure to produce an anamnestic immune response is a risk factor for development of recurrent disease (29).

In treatment of recurrent CDI, the following approach has been suggested. Diagnosis of CDI should always be confirmed by laboratory tests. The first recurrent episode should be treated as per the guidelines elaborated above according to disease severity. For a second recurrent episode a taper of vancomycin PO is recommended, which is presumed

to induce germination of spores.<sup>2</sup> For further recurrent episodes there are many suggested regimens that can include vancomycin PO (tapered or not) plus an adjunctive treatment such as cholestyramine, probiotics or IVIG (27). Alternatively, another antibiotic such as nitazoxanide or rifaximin can be trialed. In severe recalcitrant cases, fecal transplants via enema from healthy living relatives has showed anecdotal success, though this procedure carries the risk of transmitting other intestinal infections and has not been widely studied (23).

### **Prevention**

Interventions which have been shown to reduce incidence of nosocomial CDI include: timely diagnosis, cessation of offending antibiotic and treatment; prompt reporting of test results to clinicians and hospital epidemiology; discontinuation of anti-peristaltic medications when appropriate; placing the patient in isolation with contact precautions and handwashing station; and thorough cleaning of room after patient discharge with a bleach-based solution (5).

### **Diagnosis**

Despite the urgent need for early diagnosis of CDI, sensitive and specific commercially available diagnostic tests with a rapid turnaround time are lacking (30). Available test methods include:

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<sup>2</sup> The following taper regimen is suggested: 125 mg q 6 h for 7 days, then 125 mg q 12 h for 7 days, then 125 mg qd for 7 days, then 125 mg qod for 7 days, and finally 125 mg every 3 days for 7 days.

*Toxigenic Culture.* Culture of stool samples on selective, pre-reduced cycloserine-cefoxitin-fructose agar, followed by testing for the presence of toxin via ELISA or cytotoxin neutralization assay, is considered the ultimate gold standard for confirming the presence of toxigenic strains of *C. difficile*. However, it is not routinely used as a diagnostic method because it can take up to 6 days to produce results and is not always specific for toxin production in vivo. Thus, it is considered too time-consuming for clinical use (2).

*Cytotoxin Neutralization Assays (CNA).* Considered the traditional gold standard for diagnosis of CDI, these tests rely upon cell culture to detect the presence of toxin B and are generally considered to be relatively sensitive and specific (2). However, there is a great deal of variability in methodology depending upon whether or not a commercial assay is used, which cell line is employed, lab technician expertise and subjectivity in reading cell culture for cytopathic effects, and the starting dilution at which the stool samples are tested (31). In one recent study, a commonly used commercial assay (Wampole *C. difficile* Toxin B test; TechLab, Blacksburg, VA) was reported to have a sensitivity of 67.2% in comparison to toxigenic culture (32). In addition, it can take up to 48 hours to achieve results if the method is used alone and not in a two-step method. Because of the level of expertise required, CNA is only used by a minority of clinical laboratories.

*Enzyme-linked immunosorbent assays (ELISA).* For detection of toxins A and B, ELISAs are the most commonly employed tests for detection of CDI because they are relatively



cheap and quick to perform, achieving results within two hours if carried out upon immediate receipt of a stool sample. The most popular commercially available assays detect both toxin A and toxin B. A recent review by Planche and colleagues of 6 different toxin ELISAs in 18 separate studies revealed that median sensitivities of these assays ranged from 76-95% and the median specificities were 93-100%. They estimated the positive predictive value (PPV) of the various tests based upon hypothetical disease prevalence. For disease prevalence of <10% half of the assays had a PPV <80%, with that value drastically reduced with decreasing prevalence (33). At Yale-New Haven Hospital (YNHH) the disease prevalence is 10-11% based upon the number of cytotoxin positive stools received in the lab.<sup>3</sup> Just as concerning with these assays is the low negative predictive value as related to their low reported sensitivities leading to higher rates of false negative results. From a clinical perspective, this leads to repeat testing with minimal diagnostic yield and increased cost per patient (34, 35).

*Common-antigen ELISA and Two-Step Method.* The common-antigen ELISA detects the glutamate dehydrogenase (GDH) antigen that is specific to *C. difficile*. It has been shown to have a very high sensitivity (31, 36, 37), which therefore makes it an ideal screening test to confirm the presence or absence of *C. difficile*. However, it does not discern whether or not the strain is toxigenic. For this reason, this assay is commonly used in a two-step method in which GDH antigen-negative results are clinically reported as such and GDH antigen-positive results are reflexively tested by a toxin ELISA or cytotoxin neutralization assay. This method achieves relatively high sensitivity and specificity and

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<sup>3</sup> This number is an overestimation because it fails to account for repeat samples from the same patient.

can rapidly report most samples that are negative for *C. difficile*, but can still take up to 48 hours to report low cytotoxin-positive results (36-38). The two-step method is currently used for diagnosis of *C. difficile* at YNHH by the Clinical Virology Laboratory.

*Polymerase Chain Reaction (PCR)*. Real-time polymerase chain reaction (RT-PCR) assays are not widely employed for *C. difficile* infection because, prior to this study, commercial kits were not available. However, in December 2008, as this study was being completed, the Food and Drug Administration approved the first commercially available RT-PCR assay (BD GeneOhm™ Cdiff assay, BD Diagnostics, San Diego, CA) to directly detect the toxin B (*tcdB*) gene in stool to aid in the diagnosis of CDI.<sup>4</sup> Other studies evaluating PCR have only used in-house assays with variable gene targets, small numbers of samples and few positive results, making their general applicability problematic (39-45). Three prospective studies have been published to date comparing the BD GeneOhm™ Cdiff assay, a cytotoxicity assay, and toxigenic culture (32, 46, 47). Only one tested an ELISA toxin test in comparison (47). All reported the BD GeneOhm™ Cdiff assay to have a higher sensitivity than the cytotoxicity assay using toxigenic culture as the reference standard. None of the studies compared the BD GeneOhm™ Cdiff assay to a two-step testing algorithm, which is used to cost-effectively enhance sensitivity and specificity of *C. difficile* diagnosis.

Further evaluation of the BD GeneOhm™ Cdiff assay in comparison with other commonly-utilized diagnostic methods, e.g. toxin ELISA and a two-step method, is

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<sup>4</sup> Though the BD GeneOhm™ Cdiff assay had not been FDA-approved for clinical diagnostic use at the start of this study, the kits were made available to the author by BD for research purposes.

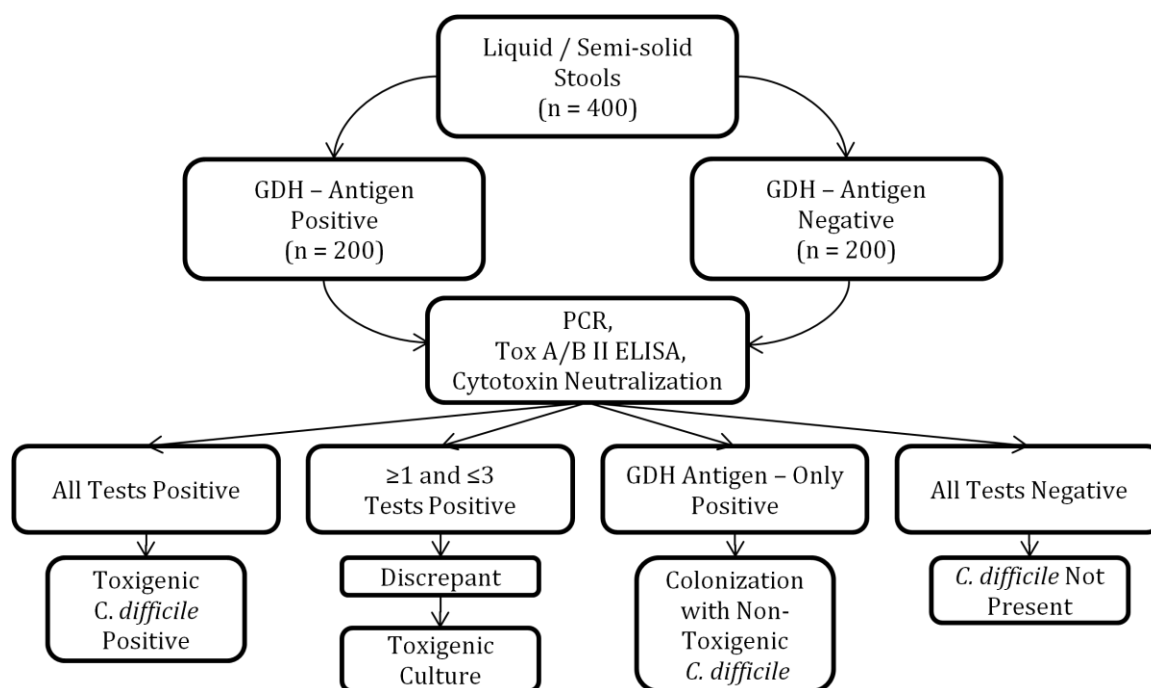
needed to assess whether or not it should be recommended for widespread clinical use. In addition, analysis of the cost-effectiveness of the BD GeneOhm™ Cdiff assay in contrast to other methods is an important factor for clinical applicability.

## **STUDY OBJECTIVES**

The objective of this study was to determine if a new commercially available PCR test would prove more sensitive and specific than standard methods for the diagnosis of CDI. The performance of the new BD GeneOhm™ Cdiff PCR assay for detection of *C. difficile* toxin B gene was compared with the two-step method currently used at YNHH (C. Diff Chek-60™ GDH-antigen assay followed by cytotoxin neutralization), and with Tox A/B II™ ELISA, the test method used in most U.S. hospitals. Toxigenic culture, the ‘gold standard’ test, was used to resolve discrepant results. The hypothesis was that PCR would prove to be the most sensitive and specific test of the clinically used methods.

## **MATERIALS AND METHODS**

***Clinical Samples.*** Liquid or semi-solid stool samples from hospitalized patients in YNHH submitted for *C. difficile* testing were entered into the study from August 2008 to December 2008. All samples were tested within 24 hours of receipt with C. Diff Chek™-60 GDH antigen ELISA, as part of the hospital’s standard two-step diagnostic routine. All *C. difficile* GDH antigen-positive with sufficient stool available, and an equivalent number of GDH antigen-negative stools were selected on each study day. All study samples were subsequently tested by cytotoxin neutralization, Tox A/B II™ ELISA, and BD GeneOhm™ Cdiff PCR Assay (**Figure 1**).

**Figure 1. Algorithm for Testing Stool Samples**

ELISA and PCR were performed by study personnel blinded to the results of the two-step method. When ELISA or PCR could not be performed on the same day, samples were frozen and thawed only once according to the manufacturers' instructions. An aliquot of each original stool sample was saved at  $-70^{\circ}\text{C}$  for further testing. Samples that did not have all four tests positive or all four tests negative were sent for toxigenic culture, excluding antigen-positive only samples. Discrepant samples from patients who were on treatment for CDI at time of sample collection were excluded from analysis. Only two samples per patient in a 7-day period were included. Repeat samples sent on the same day were excluded.

**Two-Step Method: C. Diff Chek™ -60 and Cytotoxicity Assay.** The C. Diff Chek™-60 (TechLab, Blackburg, VA) was performed according to the manufacturer's instructions. Briefly, 0.05ml of specimen was transferred to 0.2 ml of specimen diluent (buffered protein solution containing preservative<sup>5</sup>) in a 1.5 ml centrifuge tube, vortexed and then centrifuged at 5000 x g for 10 minutes. To the test microwells, 0.5 ml of conjugate solution was added, after which 0.1 ml of centrifuged specimen was added. <sup>6</sup>To the positive control well 0.1 ml of positive control (GDH-antigen) was added and to the negative control well 0.1 ml of diluent was added. The wells were covered and incubated for 50 minutes at 37°C and then they were washed in an automated washer for 7 cycles with 0.35 mL wash solution (phosphate-buffered saline and detergent). After ensuring no residual liquid was remaining, 0.1 ml of substrate (tetramethylbenzidine and peroxide) were added to each well and incubated at room temperature for 10 minutes. Then, 0.05 ml of stop solution (0.6N sulfuric acid) was added to each well and optical density measured on a microplate reader. A positive result had an optical density of  $\geq 0.080$  and a negative result had an optical density of  $< 0.080$  using the spectrophotometric dual wavelength 450/620 nm. Stool samples received before 1:00 pm were tested on the same day. Samples received after 1:00 pm were stored at 4° C and tested within 24 hours.

Stool samples positive for GDH antigen were tested by cytotoxicity assay. Stool samples (0.5 ml) were added to 0.5 ml of phosphate-buffered saline with antibiotics (vancomycin, gentamicin, and amphotericin B) and then vortexed, and the toxin was allowed to elute for 5 minutes. After centrifugation for 10 min in a microcentrifuge, the supernatant was removed and passed through a 0.45- $\mu$ m-pore-size filter. Then, 20  $\mu$ l of filtrate was

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<sup>5</sup> The preservative is 0.02% thimerosal.

<sup>6</sup> The conjugate is GDH antigen-specific mouse monoclonal antibody coupled to horseradish peroxidase.

inoculated in duplicate onto foreskin fibroblast monolayers (MRHF cells; BioWhittaker, Walkersville, MD) in 96-well plates using serial 10-fold dilutions (1:10 to 1:10,000). *C. difficile* antitoxin (20 µl; TechLab, Inc., Blacksburg, VA.) was added to one of the duplicate wells inoculated with the 1:10 and 1:100 dilutions. Thus, after addition of antitoxin, the final dilution in the first culture well was 1:20. Monolayers were read at 4, 24, and 48 hours after inoculation using an inverted microscope. A known positive control, run with each assay, was required to show cytotoxicity in the expected range. A positive result consisted of cytotoxicity that was neutralized by *C. difficile* antitoxin. Results were recorded as the highest dilution showing specific cytotoxicity. All study samples underwent the cytotoxicity assay on the same day or within 24 hours of receipt if the sample was received after 1:00 p.m.

**Toxin A/B II ELISA.** Toxin A/B II ELISA™ (TechLab, Blacksburg, VA) was performed according to the manufacturer's instructions. Briefly, 0.05 ml of specimen was transferred to 0.2 ml of specimen diluent (buffered protein solution with preservative) in a 1.5 ml centrifuge tube, vortexed and then centrifuged at 5000 x g for 10 minutes. To the test microwells, 0.5 ml of conjugate solution was added, after which 0.1 ml of centrifuged specimen was added.<sup>7</sup> To the positive control well 0.05 ml of positive control (inactivated toxins A and B) was added and to the negative control well 0.05 ml of diluent was added. The wells were covered and incubated for 50 minutes at 37°C and then they were washed in an automated washer with 0.35 mL wash solution for 7 cycles. After ensuring no residual liquid was remaining, 0.1 ml of substrate

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<sup>7</sup> The conjugate solution is a mouse monoclonal antibody specific for toxin A coupled to horseradish peroxidase and goat polyclonal antibody specific for toxin B coupled to horseradish peroxidase in a protein buffered solution.

(tetramethylbenzidine and peroxide) were added to each well and incubated at room temperature for 10 minutes. Then, 0.05 ml of stop solution (0.6N sulfuric acid) was added to each well and after 2 minutes the optical density was measured on a microplate reader. A positive result had an optical density of  $\geq 0.080$  and a negative result had an optical density of  $< 0.080$  using the spectrophotometric dual wavelength 450/620 nm. Samples not tested within 24 hours of receipt were stored at  $-20^{\circ}\text{C}$  and tested within 72 hours.

***BD GeneOhm™ Cdiff PCR Assay*** . The BD GeneOhm™ Cdiff Assay (BD Diagnostics, San Diego, CA) utilizes real-time PCR to amplify the toxin B (*tcdB*) gene in *C. difficile* and fluorogenic target-specific hybridization probes for the identification of amplified target DNA. The procedure was performed directly on stool specimens, according to the manufacturer's instructions. A sterile cotton swab was dipped into the stool specimen and then broken off into the sample buffer tube containing the Tris- EDTA sample preparation buffer that was provided by the manufacturer. The suspension in the sample buffer was vortexed at high speed for 1 min. For specimen dilution, 40  $\mu\text{l}$  of fresh sample buffer was added to a lysis tube with glass beads before transfer of 10  $\mu\text{l}$  of sample buffer containing the stool sample suspension. The lysis tube was vortexed for 5 min at high speed and pulse-spinned for 10 seconds in a centrifuge. It was then incubated in a heating block at  $95^{\circ}\text{C}$  for 5 minutes. The lysed, inactivated sample was kept on a cooling block at 3 to  $5^{\circ}\text{C}$  until testing was performed, within 30 minutes. Sample tube and reagent manipulations were performed under a ventilation hood, and reagent tubes were kept on cold blocks at 3 to  $5^{\circ}\text{C}$ . Each sample from the lysis tube (3  $\mu\text{l}$ ) was added to a SmartCycler tube containing 25  $\mu\text{l}$  of the reconstituted master mix. Included in the master mix was an Internal Control, a 333-bp DNA fragment of which only 55 bp shares

homology with *C. difficile*, to detect inhibition of the PCR. Every PCR run included a PCR-positive control (reconstituted DNA from the manufactured kit) and an uninoculated sample buffer was used as a negative control. Following centrifugation for 10 s using a Cepheid microcentrifuge especially adapted to fit the SmartCycler tubes, the reaction tubes were placed in the SmartCycler I-CORE module (Cepheid, Sunnyvale, CA) and run using Cepheid SmartCycler software with the BD GeneOhm Cdiff assay amplification protocol. Results were automatically interpreted by the software as follows: “negative,” no *tcdB* gene was detected; “positive,” the *tcdB* gene was detected; “unresolved,” either the IC was inhibited or there was reagent failure; “invalid assay run,” the PCR control (positive or negative) failed; “not determined,” there was an I-CORE module malfunction (32). Samples with unresolved results were retested from the frozen eluate after thawing. The remaining eluate in the lysis tubes was frozen at -70°C. The entire procedure required about two hours, depending on the number of samples being run. All samples not tested within 24 hours were stored at -20° C and tested within 5 days, as per manufacturer instructions.

**Toxigenic Culture.** One 2 mL vial of stool from each of the 400 samples was saved and stored at -70°C until the completion of the study, after which coded discrepant samples were tested by toxigenic culture at Montefiore Medical Center, Bronx, NY by personnel blinded to prior test results. Stool was treated with ethanol to kill non-spore flora, and inoculated in parallel onto selective cycloserine-cefoxitin-fructose agar (CCFA) supplemented with 0.1% taurocholate (Sigma, St Louis, MO) and chopped meat broth (BD BBL™, Sparks, MD) supplemented with 0.1% taurocholate, 250 µg/ml cycloserine and 16 µg/ml cefoxitin. If there was visible growth in the broth after 48 hrs, or at 5-7



days (late growth), it was subjected to the Meridian™ toxin A/B EIA (Meridian Bioscience, Cincinnati, OH). A positive result (OD >0.10) supported the detection of toxigenic *C. difficile*. Colonies of growth on the agar that had the appearance of *C. difficile* (flat yellow colonies) were tested by PCR, using an internally-validated PCR for the *tcdC* putative toxin repressor gene (5'-TCTAGCTAATTGGTCATAAG-3', 5'-AATAGCAAATTGTCTGAT-3'), as well as the GDH gene (*gdh*) using published primers [27]. All PCR reactions were performed using FastStart Hi-fidelity Taq PCR reagents (Roche, Indianapolis, IN) with MgCl<sub>2</sub> (2.5mM Mg final), on a Perkin-Elmer 2400 thermocycler, with a multiplex PCR protocol consisting of 5 min at 95°C, followed by 45 cycles of 94 °C x 1 min, 52 °C x 1 min and 72 °C x 2 min. PCR amplicons were resolved on 2% agarose gels stained with ethidium bromide. A positive ~200bp band on the *tcdC* PCR supported the detection of toxigenic *C. difficile*, and a ~750 bp *gdh* band confirmed *C. difficile*. Specimens that did not have consistent results between the toxin A/B ELISA from broth culture and toxin gene PCR had all discordant tests repeated; in addition, the broth culture was subcultured onto agar to identify individual colonies which could be analyzed by PCR. If there was a negative toxin ELISA result from broth but PCR-positive colonies on agar, the colonies were directly inoculated into broth and tested by toxin ELISA after 48 hrs.

**Discrepant Analysis.** Results were considered discrepant if 1, 2 or 3 tests were positive, excluding GDH antigen-positive only samples. Antigen-positive only samples were considered to represent colonization with non-toxigenic strains of *C. difficile*. To resolve discrepant results, three steps were taken. First, all samples for which PCR results were discordant with two-step results were repeated by PCR. Second, all discrepant samples

were submitted for toxigenic culture. Lastly, chart review was conducted for all patients with discrepant samples.

***Statistical analysis.*** Statistical analysis was performed using McNemar's test, which applies to matched pairs of dichotomous test results, e.g. when assessing the statistical significance of the observed difference between performance characteristics of two diagnostic tests (48).

**Student Responsibilities.** The author was responsible for: design of the study; submission of HIC protocol; performing BD GeneOhm™ Cdiff PCR and Tox A/B II assays on all stool specimens; performing chart review for all patients with discrepant results; analyzing results; writing the first draft of the manuscript for publication; incorporating suggestions and revisions from co-authors; and helping to revise the paper as recommended by journal reviewers. The two-step method was performed on stool samples by YNHH Virology technicians and toxigenic culture was performed by Paul Riska at Montefiore Medical Center, Bronx, NY.

## **RESULTS**

A total of 434 samples were initially tested. Of these, 18 were excluded because the patients were found to be on treatment for CDI and 16 were excluded because there were more than 2 samples per patient sent in a 7-day period. Four-hundred stool samples from 341 patients were included in the final analysis. Overall, 66 samples were positive by all four tests. Of the 200 GDH-positive samples, 71 were positive by Tox A/B II, 88 were positive by the two-step method, 96 were positive by BD GeneOhm™ Cdiff assay, and

96 were positive by GDH antigen only. For the 88 cytotoxin-positive samples in this study, 29 (33.0%) were positive at 4 hrs, 48 (54.5%) at 24 hrs, and 11 (12.5%) at 48 hrs. Six of the 11 positive at 48 hrs were positive only at the 1:20 starting dilution. Of 200 GDH-negative samples, 3 were positive by PCR only and 197 were negative for all four tests. Results are shown in **Table 1**. Two of 400 samples (0.5%) were initially unresolved by PCR but became negative upon repeat testing.

Forty-one of the samples were considered discrepant (**Table 1**) and were cultured. Thirty-nine of these were toxigenic culture-positive, and 2 were culture-negative. Fifteen cytotoxin-negative samples were GDH antigen and PCR-positive and 3 were PCR-positive only. All of these 18 were toxigenic culture positive (true positives). Twelve of 41 discrepant samples were negative by Tox A/B II and positive by all other tests, including toxigenic culture. Ten discrepant samples were cytotoxin-positive and PCR-negative and 9 were found to be toxigenic culture positive for *C. difficile*. Two of the 9 eluates, stored at -70°C, were found to be PCR-positive upon re-testing. Eight of the 9 toxigenic culture-positive samples had a very low yield of bacteria on agar culture (1+ or fewer by a semi-quantitative scoring system). Five of the 9 PCR-negative samples were cytotoxin positive at 4 or 24 hrs, and 4 were positive at 48 hrs. Chart reviews of the 9 cytotoxin-positive, PCR-negative patients revealed multiple prior or subsequent *C. difficile* cytotoxin-positive stools in 4 (44.4%) of these 9 patients. Of the remaining two toxigenic culture negative samples, one was GDH antigen and Tox A/B II-positive and the other was positive by GDH antigen and cytotoxin at 1:10 dilution only. These were designated false positives.

**Table 1. Results showing discrepant**

| No. Initial Pos Tests <sup>a</sup> | GDH Ag EIA | Cytotoxicity Assay | Tox A/B ELISA | PCR       | No. with initial results <sup>b</sup> | No. pos. by toxigenic culture | No. neg. by toxigenic culture | No. true positives <sup>c</sup> |
|------------------------------------|------------|--------------------|---------------|-----------|---------------------------------------|-------------------------------|-------------------------------|---------------------------------|
| 4                                  | +          | +                  | +             | +         | 66                                    | Not done                      | Not done                      | 66                              |
| 1                                  | +          | -                  | -             | -         | 96                                    | Not done                      | Not done                      | 0                               |
| 0                                  | -          | -                  | -             | -         | 197                                   | Not done                      | Not done                      | 0                               |
| 3                                  | +          | +                  | +             | -         | 4                                     | 4                             | 0                             | 4                               |
| 3                                  | +          | +                  | -             | +         | 12                                    | 12                            | 0                             | 12                              |
| 2                                  | +          | +                  | -             | -         | 6                                     | 5                             | 1                             | 5                               |
| 2                                  | +          | -                  | -             | +         | 15                                    | 15                            | 0                             | 15                              |
| 2                                  | +          | -                  | +             | -         | 1                                     | 0                             | 1                             | 0                               |
| 1                                  | -          | -                  | -             | +         | 3                                     | 3                             | 0                             | 3                               |
| <b>TOTAL</b>                       | <b>200</b> | <b>88</b>          | <b>71</b>     | <b>96</b> | <b>400</b>                            | <b>39</b>                     | <b>2</b>                      | <b>105</b>                      |

a. This column represents the number of tests for which any given sample was positive. For example, a sample with 4 tests positive tested positive for GDH-antigen EIA and cytotoxin neutralization assay (two-step method), Tox A/B II ELISA and PCR.

b. In total, 41 discrepant samples were tested by toxigenic culture and 39 were positive

c. A sample was considered a true positive if either all 4 tests were positive or if it was toxigenic culture positive.

At the time of the study, the BD GeneOhm™ Cdiff assay was not an FDA-cleared test, so PCR results were not clinically reported. Chart review was conducted on patients with samples that were PCR-positive but cytotoxin-negative. Eight PCR-positive, cytotoxin-negative patients were treated for CDI for a median of 1.5 days until the negative cytotoxin result was reported, and then treatment was stopped. Ten patients received no treatment. Only 5 of the 18 PCR-positive, cytotoxin-negative patients had additional testing in 6-8 months of follow-up and only one of these had a subsequent cytotoxin positive sample (22 days after the sample included in the study).

The results and performance characteristics of all tests after resolution of discrepant by toxigenic culture are given in **Table 2**. After culture resolution, Tox A/B II detected 70 (66.7%), the two-step method detected 87 (82.9%), and PCR detected 96 (91.4%) of 105 true positives. There was a 93.0% concordance of PCR with the two-step method and a 91.3% concordance of PCR with the Tox A/B II. The sensitivity, specificity, positive predictive value and negative predictive values as compared to toxigenic culture for BD GeneOhm™ Cdiff assay are 91.4%, 100%, 100% and 97%, respectively; for the two-step method are 82.9%, 99.7%, 98.9% and 94.2%, respectively; and for Tox A/B II ELISA are 66.0%, 99.7%, 98.6% and 89.4%, respectively.

**Table 2. Comparison of GeneOhm Cdiff Real-time PCR assay, a two-step algorithm and Tox A/B II ELISA after resolution of discrepant results by toxigenic culture**

| Assay                             | No. with result after resolution by toxigenic culture <sup>a</sup> |          | Performance characteristics of assay <sup>b</sup> |                 |                      |                      |
|-----------------------------------|--|----------|---|-----------------|----------------------|----------------------|
|                                   | Positive   | Negative | Sensitivity (%)                                   | Specificity (%) | PPV <sup>c</sup> (%) | NPV <sup>d</sup> (%) |
| <b>GeneOhm RT-PCR<sup>e</sup></b> |  |          | 91.4  | 100             | 100                  | 97.0                 |
| <b>Positive</b>                   | 96   | 0        |   |                 |                      |                      |
| <b>Negative</b>                   | 9  | 295      |   |                 |                      |                      |
| <b>Total</b>                      | 105  | 295      |   |                 |                      |                      |
| <b>Two-step algorithm</b>         |  |          | 82.9  | 99.7            | 98.9                 | 94.2                 |
| <b>Positive</b>                   | 87   | 1        |   |                 |                      |                      |
| <b>Negative</b>                   | 18   | 294      |   |                 |                      |                      |
| <b>Total</b>                      | 105  | 295      |   |                 |                      |                      |
| <b>Tox A/B II ELISA</b>           |  |          | 66.0  | 99.7            | 98.6                 | 89.4                 |
| <b>Positive</b>                   | 70   | 1        |   |                 |                      |                      |
| <b>Negative</b>                   | 35   | 294      |   |                 |                      |                      |
| <b>Total</b>                      | 105  | 295      |   |                 |                      |                      |

a. Samples with discrepant results were resolved by toxigenic culture.

b. Calculations make the assumption that samples with all tests positive would be toxigenic culture-positive and samples that are either GDH antigen-only positive or negative by all tests would be toxigenic culture-negative. Only a subset of GDH-negative samples were tested.

c. Positive predictive value

d. Negative predictive value

e. The difference between PCR and the two-step algorithm was not significant ( $p=0.1237$ ). The difference between PCR and Tox A/B II ELISA was significant ( $p < 0.0001$ ). McNemar's test.

The average turnaround times and materials cost per test in our laboratory, based on 5,000 samples a year are given in **Table 3**. The BD GeneOhm™ Cdiff assay is the most expensive at \$25.83 per test with the two-step method, Tox A/B II ELISA, and cytotoxicity assay following in order of decreasing cost for materials per test.

**Table 3. Comparison of time to result and materials costs for different methods**

| <b>Test</b>  | <b>Turnaround Time</b> | <b>Materials cost per test<sup>a</sup></b> |
|--|------------------------|--|
| <b>BD GeneOhm™ Cdiff assay</b>                             | 2-24 hrs               | \$25.83                                    |
| <b>Tox A/B II ELISA</b>                                    | 2-24 hrs               | \$4.12                                     |
| <b>Two-Step (GDH / Cytotoxicity Algorithm)<sup>b</sup></b> | 6-48 hrs               | \$5.02                                     |
| <b>Cytotoxicity assay only<sup>c</sup></b>                 | 4-48 hrs               | \$3.97                                     |

a, Actual costs in our laboratory. Labor, controls, repeats, are not included.

b, With the 2-step method, GDH antigen-negatives are reported within 2-24 hrs. GDH-positives are tested for cytotoxin, and cytotoxin results are reported after 4, 24 and 48 hrs of incubation.

c, Cytotoxin positives are reported at 4, 24 and 48 hrs, and all negatives are reported at 48 hrs.

## **DISCUSSION**

This is the first study comparing the performance characteristics of the commercial BD GeneOhm™ Cdiff assay to Tox A/B II and the two-step GDH antigen ELISA/cytotoxin protocol for diagnosis of CDI. The BD GeneOhm™ Cdiff assay was significantly more sensitive than the Tox A/B II (91.4% vs. 66.0% respectively,  $p < 0.0001$ ). There was an absolute increase in the number of true positives detected by BD GeneOhm™ Cdiff assay as compared to the two-step method (91.4% vs. 82.9%, respectively), but the difference

was not statistically significant in our study ( $p=0.1237$ ). The concordance of the BD GeneOhm™ Cdiff assay with the two-step method (93.0%) is similar to that of BD GeneOhm™ Cdiff assay with the cytotoxicity assay as reported by Stamper, et al (94.8%), Barbut et al (92%) and Terhes, et al (92.6%) (32, 46, 47).

All 18 two-step negative, PCR positive discrepant samples in our study were toxigenic culture positive. In contrast, Stamper et al (32) reported that 6 of 17 (35.3%) PCR-positive, cytotoxin-negative samples failed to yield toxigenic *C. difficile* on culture. Terhes, et al, (47) similarly reported 17 cytotoxin negative, PCR positive samples that were toxigenic culture positive and only 5 that were toxigenic culture negative. While this could reflect differences in the culture methods used, further study is merited.

Since PCR results were not clinically reported during our study, the 18 PCR-positive samples were reported as *C. difficile* negative and patients received brief empiric treatment or no treatment for CDI, with no apparent adverse consequences. Only one (5.5%) of 18 patients had subsequent *C. difficile* disease 22 days later. This highlights a concern for highly sensitive molecular amplification tests that target toxin genes, rather than in vivo toxin production. Patients may be colonized with toxigenic *C. difficile* but have diarrhea due to other causes. There is evidence that carriers of toxigenic *C. difficile*, who may be inadvertently identified by PCR due to inappropriate sample submission, may have immune mechanisms, which abrogate the toxin effects of their strain (4, 49, 50). The effects of eradicating asymptomatic carriage by antibiotics on the immune response of the carrier are not known. Thus, clinical correlation is even more



essential for accurate diagnosis of CDI in patients diagnosed by PCR to avoid treating patients unnecessarily (43).

It is also possible that these PCR-identified cases have low-titers of toxin production that are below the detection limits of ELISA toxin tests or cytotoxin neutralization assays. These cases might represent a small pathogen burden. In the 15-23% of CDI patients with mild disease, the symptoms may be cleared by simply removing the inciting antibiotics (22). However, these patients may still be at risk of spreading *C. difficile* spores in the hospital setting, because they test 'negative' by conventional methods, and are neither isolated nor treated. In theory, if these cases are detected by PCR, isolated and/or treated, nosocomial transmission to other more susceptible patients could be reduced. It is unclear whether or not these benefits would outweigh the costs of detecting, isolating and treating an increased number of patients with mild disease, and warrants further study.

There is some evidence to suggest correlation between host humoral immune response and severity of CDI, as mentioned previously. One study showed higher levels of antitoxin A immunoglobulin G in asymptomatic carriers of *C. difficile* than in those who developed symptomatic diarrhea (4). A later study identified a polymorphism in the interleukin-8 (IL-8) promoter gene that was associated with higher serum and fecal levels of IL-8 in hospitalized patients with *C. difficile* –associated diarrhea (CDAD) than with hospitalized controls with non-*C. difficile* diarrhea and without diarrhea (49). Higher IL-8 levels in patients were subsequently correlated with impaired levels of immunoglobulin

G to toxin A and thus enhanced susceptibility to CDAD as compared to two control groups (50). These studies support the possibility that patients who are carriers or have self-resolving, mild CDAD might have a higher level of immune response contributing to the limitation of illness. However, patients who fail to mount a significant immune response could potentially develop more severe disease manifestations from the same organism. Hypothetically, this would justify treating milder cases of CDAD that are cytotoxin negative but PCR positive. However, it should also be noted that identification of these additional patients by PCR will increase costs of isolation and prolong hospitalization. The costs and benefits of treatment for these patients merit further study.

Although PCR detected more positives than other methods in this study, 10 samples were two-step method positive and PCR-negative. Nine of these 10 samples yielded toxigenic *C. difficile* in culture and one was culture negative. Chart reviews of the 9 cytotoxin-positive, PCR-negative patients revealed multiple prior or subsequent *C. difficile* cytotoxin-positive stools in 4 (44.4%) of these 9 patients. Eight of the 9 toxigenic culture-positive samples had a very low yield of bacteria on agar culture (1+ or fewer by a semi-quantitative scoring system) and thus may have been below the detection limit of the PCR assay. This could have been due to poor initial sample quality. On retesting of the frozen PCR lysates, 2 of the 10 two-step positive/PCR negative samples were PCR positive on repeat, implying some degree of inhibition or sampling variability on initial testing. The one sample that was two-step method positive, but toxigenic culture-negative, had a low toxin titer of 1:20 at 48 hours. This was considered a false positive cytotoxin result.

Stamper et al (32) reported only one cytotoxin-positive, toxigenic culture-positive sample missed by PCR, Terhes et al (47) reported two, and Barbut et al (46) reported none. This difference might be explained by differences in cytotoxin testing methods. Cytotoxicity testing in our institution is performed by Virology Laboratory personnel, using freshly prepared human fibroblast cell culture plates, starting at a lower final dilution than most laboratories (1:20) with serial 10-fold dilutions of sample, and read at 4, 24 and 48 hours, with almost 90% of positives reported within 4-24 hours. Thus, our cytotoxin results may be superior to those obtained using higher starting dilutions of 1:50 (32) or 1:100 (46) using commercially prepared cell culture, and the BD GeneOhm™ Cdiff assay might perform even better compared to commercial cytotoxin neutralization. This reasoning applies as well to the methods for cytotoxicity assay employed by Terhes et al (47), which was initially evaluated in comparison to the BD GeneOhm™ Cdiff assay, but also used as part of their gold standard to verify toxigenicity of bacterial cultures. They utilized a HeLa cell line for cell culture, which has been shown to have a 10-fold lower sensitivity than other cell lines such as human fibroblasts used by the Yale Virology Laboratory. In addition, their cytotoxicity assay was performed up to 5 days after the receipt of the stool specimen, which may permit toxin degradation (51). The aforementioned factors are likely to greatly reduce the sensitivity of their cytotoxicity assay and, by comparison, make the performance of the BD GeneOhm™ Cdiff assay appear artificially elevated. They report a sensitivity and specificity of 96.4% and 99.1% respectively (47), as compared to 83.6% and 98.2%, respectively, by Stamper et al (32) and 91.4% and 100.0%, respectively, in our study.

Nevertheless, the failure of PCR to detect 9 true positives detected by the two-step method was a concern. It is unlikely that the BD GeneOhm Cdiff assay PCR failed to detect these 9 samples due to inhibitors for several reasons. First, each reaction mix has an internal amplification control to monitor reagent integrity and PCR inhibition. Inhibitory samples have an internal control result of “FAIL” and a BD GeneOhm Cdiff assay result of “Unresolved”. The 9 samples passed the test for internal control amplification. Secondly, eight of the nine samples had very low yield of *C. difficile* in direct anaerobic culture, with <10 cfu<sup>8</sup> recovered for 5 of those samples. In addition, two of the stool samples were positive on repeat testing of the sample lysates, suggesting sample variability in the runs. These findings support the idea that the inability of the BD GeneOhm Cdiff assay to detect these samples was more likely due to a bacterial load below the level of detection of the assay, suggesting that dilution of the samples will not likely improve PCR detection. The quality of the stool sample at initial collection is more likely to be the source of low bacterial load.

In addition to low numbers of bacteria and presence of inhibitors, another possible explanation for the failure of the BD GeneOhm™ Cdiff assay to detect samples that were positive by the two-step method is genetic variance at the *tcdB* locus, leading to mismatch of PCR primers. While the majority of isolates of toxigenic *C. difficile* come from toxinotype variants with an intact *tcdB* gene, mutations and deletions in *tcdB* have been documented (12, 52). A recent study discovered a genotype of a binary toxin-producing *C. difficile* strain that contained the toxin A and binary toxin genes, but was

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<sup>8</sup> Colony forming units.

negative for the toxin B gene (53). This strain is genetically distinct from the epidemic BI/NAP1/027 strain. However, reports of such toxigenic *C. difficile* variants are very rare, as toxin B appears to be an essential virulence factor (54). Preliminary unpublished data from Paul Riska shows that, in testing over 70 isolates of each type, the BD GeneOhm PCR is equally efficient at detecting the current North American epidemic (ribotype 27) and non-epidemic strains. However, monitoring for evolution of new variants of *tcdB* is warranted.

The Tox A/B II ELISA failed to detect 35 toxigenic culture-positive samples and yielded one false positive result, consistent with the documented low sensitivities of this class of tests (33, 55). Cytotoxin neutralization as performed in our virology laboratory, detected 9 true positives missed by PCR. However, due to the techniques employed at YNH mentioned above to maximize sensitivity of the cytotoxicity assay, it may be that our lab is an outlier in the quality of this test. Other labs that solely utilize an ELISA toxin assay or a commercial cytotoxin neutralization assay with much lower sensitivity than ours could be missing many true positive cases of CDI that would be detected by the BD GeneOhm™ Cdiff assay.

The BD GeneOhm™ Cdiff assay was simple to perform and produced results in approximately 2 hours, compared to 2 hours for the Tox A/B II ELISA and 6-48 hours for the two-step method (**Table 3**). The PCR method is more expensive than other methods, but may reduce nosocomial transmission of toxigenic *C. difficile*, and thus lead to long-term savings for hospitals and patients. Of note, the costs for each test will vary

among institutions depending on test volume, contracts with suppliers, and other factors. In our virology laboratory, cytotoxin neutralization is an inexpensive test. Thus, to test 5,000 samples a year using BD GeneOhm™ Cdiff assay as the sole assay will cost an additional \$100,000 in reagents, which is a significant barrier to implementation. In addition, the increased number of cases detected per year by PCR will require isolation and treatment, resulting in increased hospital costs over the short term. Yet, in the long-term, these costs could be offset by reduction in the overall rates of transmission of *C. difficile* spores if more ‘true positives’ are isolated and treated. Moreover, more rapid diagnosis of cases would result in earlier isolation and presumably decreased nosocomial transmission. For instance, at YNHH the two-step diagnostic algorithm can take up to 48 hours to receive a positive result for CDI, during which time the patient is not placed on isolation precautions. If PCR were used a positive result theoretically would be available within hours 2- 24 hours after the sample was submitted to the lab.

Chart review of patients with discrepant samples revealed hospital-wide problems with stool sample submission for *C. difficile* analysis, including submission of samples from patients with minimal diarrhea, from patients who were already on treatment, and multiple samples from the same patient on the same day or within a 7-day period. Though discordant samples from patients found to be on therapy were excluded from analysis in the study, these problems were illustrative of inappropriate clinical use of the test, which ultimately drives up overall health care costs. To both avoid unnecessary treatment and reduce costs of PCR testing, clinicians will need to be educated about limiting *C. difficile* testing to patients with a reasonable probability of having disease, such as those patients

having 3 or more loose stools per day for 1 to 2 days (43). Moreover, clinicians should not be ordering *C. difficile* testing for patients who are undergoing antibiotic treatment for CDI as this can interfere with the toxin gene expression and increase the likelihood of false negative test results. Utilization of automatic clinician reminders or ‘pop-up’ windows through computerized electronic ordering systems would be one way to reduce inappropriate ordering of these tests, and thus decrease overall costs associated with the disease.

There were several limitations of this study. Only the discrepant samples were cultured and not all samples tested were included in the study. The performance characteristics of the various assays were compared to toxigenic culture as a gold standard, with the assumption made that samples for which all four tests were positive would be toxigenic culture-positive and those for which all four tests were negative would be toxigenic culture-negative. All GDH-antigen positive samples submitted on study days that met study criteria, but only a subset of GDH-antigen negative samples, were included. In addition, samples were sent to a separate institution for toxigenic culture and different assays were used to determine whether strains of *C. difficile* were toxigenic. Specifically, a Meridian toxin A/B EIA and an in-house PCR assay to detect *tcdC* (toxin repressor gene) were utilized, rather than the TechLab toxin A/B ELISA and the *tcdB*-based PCR assay used in the initial analysis. Thus, some of the divergent results may be due to different performance attributes of these particular assays. However, it is likely that a greater difference was attributable to the fact that the toxigenic culture assays were applied to an amplified culture rather than a crude stool sample.

In conclusion, the BD GeneOhm™ Cdiff assay is the first FDA-approved commercial PCR kit for diagnosis of toxigenic *C. difficile* and could be a promising new tool. This study found it to be much more sensitive than a common ELISA toxin test for diagnosis of toxigenic *C. difficile*, which is the most widely used class of diagnostic assay for *C. difficile* infection, yet typically has poor sensitivities. The BD GeneOhm™ Cdiff assay is as sensitive and specific as a two-step method, currently used at YNHH. It is more expensive, but has a faster turnaround time than the two-step method, which could lead to earlier diagnosis of CDI and reduction of nosocomial transmission, hypothetically resulting in overall cost savings. A potential concern is increased treatment of asymptomatic carriers or mild, self-resolving disease. This study also found inappropriate utilization of the two-step method to be quite common at YNHH, including ordering the test multiple times for one patient on the same day or within a 7-day period, and when the patient was being treated for CDI. The implementation of any diagnostic test should be accompanied by education of clinicians about appropriate use of the test and interpretation of its results



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