Comparing Immuno*Card* with Two EIA Assays for *Clostridium difficile* Toxins

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OBJECTIVE: To compare three *Clostridium difficile* EIA kits for the detection of *C. difficile* toxins from clinical specimens.

DESIGN: A total of 287 fresh and stored stool specimens were tested using all three assays. Stools with discrepant results were sent to a reference laboratory for tissue cytotoxin assay.

SETTING: Trinity Medical Center, a community hospital with network hospitals

PATIENTS: Patients with diarrhea submitted stools for detection of *C difficile* toxins

RESULTS: Of the 287 stool specimens, 116 were positive and 171 negative for *C. difficile* toxins. The sensitivity, specificity, and positive and negative predictive values of Meridian EIA assay were 99.1, 97.7, 96.6, and 99.4%; Immuno*Card* were 100, 98.2, 97.5, and 100%; BioStar OIA assay were 94, 98.8, 98.2, and 96% respectively. Immuno*Card* provides the best sensitivity (100%) for *C.difficile* toxins A and B detection. The BioStar OIA rapid test missed seven positive stool specimens possibly due to failure to detect toxin B.

CONCLUSION: Immuno*Card* has slightly higher predictive values, shorter turnaround time and greater convenience compared to the Meridian EIA Assay. Immuno*Card* may be cost effective not only in smaller laboratories, but also in high volume laboratories, when used on a STAT basis or single request.

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ABBREVIATIONS: *C. difficile* = *Clostridium difficile*, EIA = Enzyme Immuno Assay

INDEX TERMS: *C. difficile* toxins; method comparison

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INTRODUCTION

Clostridium difficile, a spore forming Gram-positive anaerobic bacterium, is the major causative agent of colitis and diarrhea that may occur following antibiotic therapy[1]. *C. difficile* is acquired primarily in hospitals and chronic care facilities and represents one of the most common worldwide nosocomial infections[2]. The organism can be cultured from bed rails, toilets and the floors of the rooms of the patients suffering with *C. difficile*-associated diarrhea, as well as from the hands of health care workers caring for the patients[3]. Almost all the patients have been treated with antibiotics or chemotherapy within eight weeks prior to the onset of diarrhea. Many anti-

biotics have been implicated in this process; clindamycin and third generation cephalosporins are the leading antibiotics in most cases[4]. It is postulated that the administration of broad-spectrum antibiotics causes the disruption of normal gut flora, after which colonization with *C difficile* occurs by ingestion of spores from contaminated objects. Depending on the host's immune status, patients may become asymptomatic carriers or develop diarrhea or pseudomembranous colitis[5]. *C difficile* also causes outbreaks of intestinal disease in hospitalized patients. One study documented 176 *C difficile* outbreaks in England and Wales during 1992 to 2000, 12.6% of the total infections[6].

Toxigenic *C difficile* elaborates two toxins (toxin A and toxin B) during multiplication in the intestinal lumen. Toxin A binds to the colonic mucosa to induce an inflammatory response through the activation of macrophages and mast cells, which leads to fluid secretion and increased mucosal permeability[7,8]. Toxin B causes depolymerization of filamentous actin and is extremely cytotoxic in vitro[8,9]. Both toxins are responsible for the pathogenesis of diarrhea and colitis. Another toxin, a binary toxin may also be an additional virulence factor of the organism. Its role in the pathogenesis is being investigated[10].

Laboratory diagnosis of *C. difficile* infection is achieved through the detection of toxins in the stool of suspected patients. The tissue cell culture cytotoxin assay is considered the gold standard for toxin B detection, due to its high specificity and sensitivity at the picogram level. The assay is time consuming and technically demanding, requiring a facility capable of performing cell culture and takes 48 hours to complete. Over the years, the more rapid EIA assays have largely replaced the cytotoxin assay. Early EIA assays detected only toxin A, while the newer EIA assays detect both toxins A and B. EIA assays using the microtiter plate format are aimed at high volume laboratories, while the rapid point-of-care type lateral flow assay will be cost effective for low volume laboratories. The Meridian EIA is a relatively rapid test that takes approximately 60 minutes, detects both toxins A and B in stool specimens, and is geared for high volume laboratories. BioStar OIA is a rapid assay (15 minutes) that detects only toxin A. Immuno*Card* is a reformulated rapid assay similar to the BioStar OIA, and detects both toxins A and B in stool specimens.

In this study, we compared the diagnostic values of the BioStar OIA and the Immuno*Card* with our current laboratory assay, the Meridian EIA. This study included stored positive specimens to increase the positive rate in order to bring out the statistical significance of each assay and their predicative values. We also evaluate the utility of Immuno*Card* in both a low and a high volume laboratory setting.

MATERIALS AND METHODS

A total of 287 fresh and stored stool specimens from patients collected during a six-month period at Trinity Medical Center, Princeton Baptist Medical Center and other Baptist Hospitals within the Baptist Health Care System of Alabama were tested by all three methods (Meridian EIA, Immuno*Card* and BioStar OIA). Stored specimens were aliquoted and frozen at -20°C upon arrival in the laboratory. In order to preserve the integrity of the toxins, all fecal specimens were thawed only once for testing purposes. Some stored specimens were previously tested as positive by other laboratories and were sent to Trinity Medical Center to increase the number of positive specimens for the study. All specimens were handled and tested by all three methods according to the manufacturer's recommendations. The technologists who performed the test were blind to the test results of the other tests. If all three methods demonstrated the same results, no further testing was performed and the results were considered true positive or true negative. Any discrepant results between the three methods were considered to be indeterminate. An aliquot of the specimen from the freezer was subsequently sent to a reference laboratory (Trihealth Laboratories; Cincinnati, Ohio) for the tissue cell culture cytotoxin assay.

Testing was performed and interpreted according to the manufacturer's instructions. Briefly, the procedures are as follows:

Meridian EIA: The diluted stool (100µl) was mixed with 50µl of the Enzyme Conjugate, incubated for 50 minutes at 35-39°C, washed and 100µl of substrate added. It was incubated another 10 minutes, stop solution was added, and the results read within 15 minutes at a wavelength of 450nm, resulting in a total assay time of slightly over one hour.

Immuno*Card* Toxins A & B: The stool sample was added to a mixture of Specimen Diluent and Enzyme Conjugate and incubated five minutes at 20-26°C. Then 150µl of diluted specimen was added to each of the two sample ports on one test card, incubated for five minutes at 20-26°C and washed. The substrate was added and the test card incubated at 20-26°C for another five minutes. The results were read visually within 30 seconds of the end of the incubation period. Positive test result is demonstrated by

the development of blue color in the "TEST" and "CONTROL" reaction ports. The total time for the assay was about 15 minutes.

For the BioStar OIA the stool sample was mixed with the substrate, two drops of sample mixture were added to the center of the test surface. After five minutes the surface was washed and one drop of substrate was added. After another five minutes the module was washed and the test surface was evaluated for a color change, resulting in a total time expenditure of approximately 15 minutes.

The sensitivity, specificity, and positive predictive value and negative predictive value of each method were calculated based on the final results. The results were subjected to statistical analysis using Fisher's exact test.

RESULTS

Of the 287 specimens, 116 were positive and 171 were negative for the *Clostridium difficile* toxins. 109 specimens were positive and 163 were negative by all three assays. The sensitivity, specificity, and positive and negative predictive values of Meridian EIA assay were 99.1, 97.7, 96.6, and 99.4%. Immuno*Card* 100, 98.2, 97.5 and 100%. The BioStar OIA assay 94, 98.8, 98.2 and 96% respectively (Tables 1 and 2). The 95% Exact Confidence Intervals between the assays were shown in Table 3.

All three assays require very little specimen preparation and the procedures are simple to follow. The Meridian EIA has a longer incubation time, requiring 60 minutes to complete the assay, and requiring one positive and one negative control for each run. Both the BioStar OIA and the Immuno*Card* require only 15 minutes from specimen preparation to the reading of results. Both assays contain internal controls for validation of the assay.

Table 1. Comparison of Meridian EIA assay, Immuno*Card* and BioStar OIA

Method	Sensitivity %	Specificity %	PPV %	NPV %
Meridian EIA	99.1	97.7	96.6	99.4
Immuno*Card*	100	98.2	97.5	100
BioStar OIA	94	98.8	98.2	96

Table 2. Actual Raw Numbers.

Method	True Positive	False Positive	False Negative	True Negative
Meridian EIA	115	4	1	167
Immuno*Card*	116	3	0	168
BioStar OIA	109	2	7	169

DISCUSSION

The tissue cytotoxin assay is currently the widely accepted gold standard diagnostic method for laboratory diagnosis of *Clostridium difficile*-associated disease. However, it has a relatively long turnaround (TAT) time (48 hours) and requires a tissue culture facility. Although the cytotoxin assay is highly sensitive for detection of toxin B, it is not standardized and the procedures and the cell lines used vary between laboratories[11]. Because of the complexity and time requirements of the cytotoxin assay, multiple commercial immunoassays have been developed for the detection of toxin A /and toxin B. Turgeon[12] used more than 1000 fecal samples to compare six commercially available immunoassays for *Clostridium difficile* toxins with the cell culture cytotoxin assay. They demonstrated that the assays which detected both toxin A and B had the best overall performance among the toxin-only test, having the highest positive predictive value and the second highest negative predictive value. Their data also suggested that the single-use card format is inferior to traditional enzyme-linked immunosorbent assays. The two single-

use card assays in their study were Triage Micro *C. difficile* (Triage, BioSite Diagnostics) and the original ImmunoCard *Clostridium difficile* (I Card; Meridian Bioscience) both detected toxin A and the common antigen in combination or separately. Others also reported the lack of sensitivity of this original Immuno*Card* (toxin A only) when compared with cytotoxin assay and toxigenic culture[13].

In this study, we evaluated the Meridian reformulated Immuno*Card,* and compared it with the Meridian regular EIA and BioStar OIA assays. The Immuno*Card* has been reformulated to include both toxin A and B, but no common antigen. It is still in a single-use lateral flow card format immunoassay with a turnaround time (TAT) of about 15 minutes. The BioStar OIA, which detects toxin A only, is another commercially available single-use lateral flow card immunoassay that has a similar TAT as the ImmunoCard. The Meridian EIA is our regular EIA assay that detects both toxin A and B in a 96 microwell format. The TAT is about 60 minutes, but somewhat longer for high volume laboratories.

The Immuno*Card* offers the advantage that it is the first introduced point of care *C. difficile* assay that can detect both toxin A and B with a TAT of about 15 minutes. Several months after initiation of this study, Remel introduced a similar rapid assay that can also detect both toxin A and B. Unfortunately, our study was already underway and we were not able to include the Remel kit in this study. The Immuno*Card* is a truly rapid assay that has a TAT of 15 minutes, and is less labor intensive than the Meridian EIA assay (60 minutes TAT). The BioStar OIA, is also very simple to perform with a short TAT. Moreover, the differences between the Immunocard and the BioStar OIA were statistically significant (Table 3). Our laboratory policy does not accept formed stools, and therefore did not have the same slow-flow problems as reported in the original Immuno*Card* assay[13], but care should be taken to avoid inundation of the Immuno*Card* assay with too much stool specimen.

Table 3. 95% Exact Confidence Intervals

	Sensitivity	Specificity
Meridian EIA	95.3 - 99.9*	94.1 - 99.4
Immuno*Card*	96.9 – 100.0**	95.0 – 99.6
BioStar OIA	88.0 – 97.5	95.8 – 99.9

* p=0.066 versus BioStar OIA, Fisher's Exact Test.
** p=0.014 versus BioStar OIA, Fisher's Exact Test.

Of the 287 specimens tested, 109 (37.9%) were positive and 163 (56.8%) were negative by all three assays, giving a total agreement of 94.8% (272 of 287). BioStar OIA had the least sensitivity (94%) of the three assays in this study. The BioStar OIA missed seven positives (6%). The low sensitivity of the OIA assay in comparison with the other two assays could be because this assay missed specimens that contained toxin B only. McGowan and Kader[14] used a toxin A only assay in a pediatric population that resulted in positives only 50% of the time.

Nosocomial outbreaks caused by toxin A deficient (A- B+) *Clostridium difficile* strains that resulted in the death of patients in Canada and US have been reported[15,16,17]. This emphasizes the importance of the detection of toxin B in patient stool specimens. The incidence of toxin A negative, toxin B positive can be between 17 to 34% as noted in one study in NJ hospitals[18]. Thus, BioStar OIA may not be a viable alternative diagnostic method for the detection of *Clostridium difficile* toxins.

The Immuno*Card* performed quite differently from the original assay[12,13]. The original assay performed similarly to other lateral flow assays that have poor sensitivity as well as missing toxin B when compared with their regular Meridian EIA assays[12]. This new assay can detect both toxin A and B, and the reformulation probably improved the sensitivity of the assay. In our hands, it performed slightly better than our regular Meridian EIA, with 100% sensitivity and NPV (Table 1) and a TAT of 15 minutes. This performance is as good, if not better than the Meridian EIA assay. For low volume laboratories, the Immuno*Card* can be used as their regular assay without compromising performance.

Rapid single cartridge assays are not ideal for high volume laboratories because of the cost and labor requirements per test compared to batch testing. However, the Immuno*Card* assay can play a significant role in high volume laboratories when a rapid result is needed. The TAT of 15 minutes makes this test an excellent assay for a STAT situation. This kit may also be cost-effective for weekends when only one or two requests are received. The cost of the Immuno*Card* is about $600 for a kit of 50 tests, making it $12 per reportable (with built in internal controls). The regular Meridian EIA with two controls will cost $12.50 for one specimens, $16.67 ($8.33 per reportable) for two specimens and $20.83 ($6.94 per reportable) for three specimens. The Immuno*Card* is cost

effective for one specimen and probably for two specimens, depending on the cost of technologist time.

There are limitations in our study. First, this is not a true prospective study. The positive rate in this study is much higher than most hospital's prevalence rate. We purposely increased our positive specimens in order to enhance the statistical calculation of each assay. A low prevalence rate would require a much larger sample size in order to detect the difference between these three assays. This high prevalence rate does not reflect the actual incident of most hospitals, but will enhance the differentiation of the three assays. That is what we intended to do. Secondly, our laboratory is not equipped to do cytotoxin assay. To send out all 287 specimens for cytotoxin assay would be beyond what our budget could afford. Therefore we chose to use the combination of consensus and cytotoxin assay to be our gold standard. For positives, we relied on specimens that were positive by any one of the three assays. If a discrepancy occured between these assays, we then used cytotoxin assay to determine if the specimens were truly positive. Thus the sensitivity, specificity and predicative values were calculated comparing the three assays evaluated. For practical purposes, the sensitivity and specificity in this study is between these three assays

In conclusion, Immuno*Card* detects both toxin A and B from patient stool specimens with similar performance to the regular Meridian EIA. The assay can be completed in 15 minutes, making it an excellent assay for low volume laboratories. This assay may be effective not only in smaller laboratories, but also in high volume laboratories. Due to its single use card format, it may also be cost-effective when used on a STAT basis on weekends when only one or two are requested. Laboratories should not use an assay that detects only toxin A due to poor sensitivity.

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