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Evaluation of Methods for Detection of *Clostridium difficile* and its Toxins in Patients with Nosocomial Diarrhoea

[1,2]N. Sadeghifard, [1]M.H. Salari, [1]M.R. Ghasemi and [1]F. Amin-Harati
[1]Division of Bacteriology, Department of Pathobiology, School of Public Health,
Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

Abstract: The aim of this study was to compare stool cytotoxin assay as the standard test with enzyme immunoassay system (*C. difficile* Tox A/B Tech Lab) and *C. difficile* bacterial culture on cycloserine-cefoxitin fructose agar followed by cytotoxin assay (toxigenic culture). A total of 650 stool specimens from hospitalized patients with nosocomial diarrhoea were collected from December 2002 to February 2004. *C. difficile* was detected by culture method in 146 samples. Of these samples 103 isolates were toxigenic by toxigenic culture. One hundred twelve and 108 stool specimens were positive for *C. difficile* toxins by enzyme immunoassay (Tox A/B immunoassay) and stool cytotoxin assay, respectively. Ninety eight samples were diagnosed to be positive in all test methods. Toxigenic *C. difficile* was isolated from 2 stool cytotoxin assay and 3 enzyme immunoassay negative stools. Sensitivity and specificity for Tox A/B immunoassay in relation to the stool cytotoxin assay was 95.6% (95% CI) and 98.3% (95% CI), respectively. The results of the present study showed that sensitivity and specificity Tox A/B immunoassay was very close with stool cytotoxin assay.

Key words: *Clostridium difficile*, stool cytotoxin assay, enzyme immunoassay, toxigenic culture

INTRODUCTION

Clostridium difficile is one of the most frequently identified causes of nosocomial gastrointestinal disease[1]. It has been implicated as a causative agent in antibiotic-associated diarrhea, antibiotic-associated colitis and pseudomembranous colitis[2,3]. *C. difficile* associated-diarrhea (CDAD) is most often associated with nosocomial acquisition and prior antibiotic therapy, but the immunocompromised state (due to factors other than prior antibiotic therapy), bowel surgery and bowel stasis are also predisposing factors[2,3]. CDAD may also occur when no known risk factors are present[4]. Many strains of *C. difficile* produce two protein exotoxins, A and B, which are thought to be the primary cause of colonic mucosal injury and inflammation[2,5]. Toxin A exerts primarily enterotoxic effects, while toxin B is primarily cytopathic effect.

The biological diagnosis of digestive tract infections associated with *C. difficile* is based either on the isolation of the bacterium or on the detection of toxins (A or B) in fecal samples[6]. At present, the reference method is the stool cytotoxin assay, which reveals the presence of toxins by the cytopathic effect in cell cultures[7]. However, this method is time-consuming (it takes at least 24 h), it requires facilities for cell culture testing and it needs an antitoxin to confirm the specificity of the observed cytopathic effect. The method also lacks standardization. During the last 15 years, several enzyme immunoassays have become commercially available, detecting either toxin A or both toxin A and toxin B[8,9].

The aim of this study was to compare stool cytotoxin assay as the standard method with enzyme immunoassay system for detect Tox A and B (C. difficile Tox A/B II Tech Lab, Inc., Blacksburg, Va) and culture for the organism with follow-up toxin testing (toxigenic culture).

MATERIALS AND METHODS

Study site and stool specimens: During the study period (from December 2002 to February 2004), 650 stool samples from hospitalized patients (between 2-80 years) were screened for presence of *C. difficile* and its toxins. These 650 samples, from 3 tertiary-care hospitals (Emam khomeini, Shariatei and Children's Medical Center) in Tehran-Iran, fell into two groups. The first group consisted of samples for which the clinicians had specifically requested examination for *C. difficile* toxin (290 samples) and the second group consisted of

Corresponding Author: N. Sadeghifard, Division of Bacteriology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Poursina St, Tehran, Iran Tel: +0 98 9125146874

360 stool samples, which were selected by laboratory criteria. All of stool samples were tested by three techniques include stool cytotoxin assay for the presence of toxins in stool, as the standard method, culture for the organism with follow-up toxin testing (toxigenic culture) and enzyme immunoassay system for detect Tox A and B (*C. difficile* Tox A/B II Tech Lab, Inc., Blacksburg, VA.). All stool samples were examined with three techniques just for one time and all results had been included in final analysis.

The selection criteria were: long stay hospitalization (>five days), loose, liquid stools (bloody and/or mucoid), lack of other enteric pathogenic bacteria, viruses, ova or parasites and the fact that the clinicians had not requested *C. difficile* toxin examination. Specimens were processed immediately (the day of receipt) or stored at -20°C until they were tested.

Stool cytotoxin assay: A filter-sterilized (0.45 μ), 1:10 dilution of feces was used to inoculate Vero cell monolayer (including growth medium eagle minimal essential medium supplemented with 2% fetal bovine serum) with and without neutralizing *C. difficile* antitoxin (Tech Lab). Tissue cultures were examined at 24 and 48 h. Characteristic Cytopathic Effect (CPE) in more than 50% of cells across the cell sheet that neutralized by antitoxin was interpreted as a positive result. Where a cytopathic effect was observed with a 1:10 dilution of feces and was neutralized by antitoxin, the assay was repeated using higher dilutions (1:40 and 1:100) of feces (the lower dilution was used for screening and the higher dilution of stool used for confirmation of the test).

Culture for the organism with follow-up toxin testing (toxigenic culture): A portion of each specimen were treated with alcohol for spore selection and cultured on cycloserine-cefoxitin fructose agar (Mast, UK) for isolation of *C. difficile*. This medium was supplemented by 10% horse serum, cycloserine (250 mg L^{-1} and cefoxitin (8 mg L^{-1}). For alcohol shock one volume of stool was diluted with approximately equal volume of Brain Heart Infusion Broth and mixed with twice the volume of ethanol 95%. After incubation for 30 min at room temperature, with cotton swab was inoculated to cycloserine-cefoxitin fructose agar. Plates in an anaerobic chamber were incubated for 48 h at 37°C. Colonies that were suspected of being *C. difficile* on the basis of characteristic morphology, odor and gram stain morphology were identified using conventional biochemical methods[10]. All isolates were negative for lipase, lecithinase and indole production as well as for milk digestion. These isolates fermented glucose and mannitol but did not ferment maltose and sucrose. For toxin assay 3-6 colonies of *C. difficile* was inoculated in Brain Heart Infusion Broth (Oxoid, UK), in an anaerobic chamber for 5-7 days at 37°C. Broths were centrifuged for 10 min at 2500× g and the supernatant was filtrated (0.2 μ) and Vero cell monolayer in micro titer plates were inoculated with 100 μL of culture filtrates which were prepared by 10 fold serial dilution in Eagle minimal essential medium supplemented with 2% fetal bovine serum (Gibco). Tissue cultures were examined at 24 and 48 h. Characteristic Cytopathic Effect (CPE) neutralized by *C. difficile* antitoxin (Tech Lab) was interpreted as a positive result.

***C. difficile* Tox A/B Immunoassay (Tech Lab):** The fecal samples were tested directly for Toxins A and B by the manufacturer's recommended procedures. This kit is formatted to simultaneously detect *C. difficile* toxins A and B. The *C. difficile* Tox A/B II is an ELISA and is an alternative to tissue culture assay for detecting *C. difficile* toxins in fecal specimens. The test is completed within 1 h. The *C. difficile* Tox A/B II test uses antibodies to *C. difficile* toxins A and B. The microassay wells supplied with the kit contain immobilized affinity-purified polyclonal goat antibody against toxins A and B. The detecting antibody consists of a mixture of toxin A monoclonal mouse antibody conjugated to horseradish peroxidase and toxin B polyclonal goat antibody conjugated to horseradish peroxidase. In the assay, an aliquot of a fecal specimen is emulsified in the Diluent and the diluted specimen is then transferred to the microassay well containing the detecting antibody. If toxins A and B are present in the specimen, they will bind to the detecting antibody and to the immobilized polyclonal antibody during the incubation phase. Any unbound material is removed during the washing steps. Following the addition of substrate, a color is detected due to the enzyme-antibody-antigen complexes that form in the presence of toxin.

Statistical methods: Data were validated and analyzed using Statistical Package for Social Sciences Software.

RESULTS AND DISCUSSION

During December 2002 to February 2004, 650 stool samples were collected from 650 patients, that 340 (52.3%) of the patients were male and 310 (47.7%) were female. 370 (56.9%) patients were admitted at Children's Medical Center, 175 (26.9%) patients were at Emam Khomeini Hospital and 105 (16.2%) patients admitted at Shariatei Hospital.

Table 1: Results of culture of organism, toxigenic culture, stool cytotoxin assay and enzyme immunoassay (Tox A/B Tech Lab)

Results	Culture	Toxigenic culture	Stool cytotoxin assay	ToxA/B immunoassay
Positive	146	103	108	112
Negative	504	547	542	538
∑	650	650	650	650

Table 2: Comparison of stool cytotoxin assay results with Tox A/B immunoassay results

		Stool cytotoxin assay results		Performance characteristics (%) (95% CI)			
Test method	Results	Positive	Negative	Sensitivity	Specificity	PPV*	NPV†
Tox A/B immunoassay	Positive	103	9	95.6	98.3	92.3	99.1
	Negative	5	533				
	∑	108	542				

* Positive predictive value, † Negative predictive value

Table 3: Comparison of stool cytotoxin assay results with toxigenic culture

		Stool cytotoxin assay results		Performance characteristics (%) (95% CI)			
Test method	Results	Positive	Negative	Sensitivity	Specificity	PPV*	NPV†
Toxigenic culture	Positive	101	2	93.9	99.6	98.2	98.7
	Negative	7	540				
	∑	108	542				

* Positive predictive value, † Negative predictive value

Table 4: Comparison of stool cytotoxin assay results with culture method

		Stool cytotoxin assay results		Performance characteristics (%) (95% CI)			
Test method	Results	Positive	Negative	Sensitivity	Specificity	PPV*	NPV†
Culture	Positive	101	45	93.9	92.3	70.5	98.7
	Negative	7	497				
	∑	108	542				

* Positive predictive value, † Negative predictive value

Among the 650 fecal samples tested in 146 samples (22.46%) *C. difficile* was isolated by culture method. Of these samples 103 (15/84%) were positive by toxin testing (toxigenic culture). One hundred eight (prevalence: 16.6%) and 112 stool samples were positive by stool cytotoxin assay and enzyme Immunoassay (Tox A/B II Tech Lab), respectively (Table 1). One hundred seventeen specimens were positive by one or more of the three methods and 98 samples were positive in all of tests. Both stool cytotoxin assay and toxigenic culture produced positive results for 101 samples. In addition, 43 strains were isolated from fecal samples that were not toxigenic by toxin testing (toxigenic culture). A total 533 samples were negative in all assay systems. Comparisons between these techniques (Table 1-4) showed that toxigenic *C. difficile* was isolated of three Tox A/B immunoassay negative stools and two stool cytotoxin assay negative stools. Sensitivity, specificity, positive predictive value and negative predictive value for toxigenic culture in relation to the stool cytotoxin assay was 93.9% (95% CI), 99.6% (95% CI), 98.2% (95% CI) and 98.7% (95% CI), respectively (Table 3).

The present study was undertaken to compare stool cytotoxin assay as the standard method with toxigenic culture and Tox A/B immunoassay. Comparison between stool cytotoxin assay with Tox A/B immunoassay (Table 2) showed that 5 fecal samples that tested positive with stool cytotoxin assay, were not detected by Tox A/B immunoassay (false-negative results) and 9 fecal samples that tested negative with stool cytotoxin assay, tested positive with the Tox A/B immunoassay (false-positive results).

In this study sensitivity, specificity, positive predictive value and negative predictive value for Tox A/B immunoassay in relation to the stool cytotoxin assay was 95.6% (95% CI), 98.3% (95% CI), 92.3% (95% CI) and 99.1% (95% CI), respectively (Table 2). According to the study of Turgeon *et al.*[11] that was undertaken on the six rapid tests for direct detection of *C. difficile* and its toxins, sensitivity and specificity for Tox A/B immunoassay compared to stool cytotoxin assay was 77.2 and 99.4%, respectively which sensitivity is relatively different with the results in this study. In the other study, O'Connor *et al.*[10] worked on evaluation of methods for detection of toxins, sensitivity and specificity for Tox A/B immunoassay in comparison to stool cytotoxin assay was 80 and 99%, respectively.

Comparison between stool cytotoxin assays with toxigenic culture (Table 3) showed that 2 fecal samples that tested positive with toxigenic culture were not detected by stool cytotoxin assay and 7 samples that tested negative with toxigenic culture, tested positive by

stool cytotoxin assay. The major objection made against the use of toxigenic culture as a diagnostic tool is that toxigenic culture positive and stool cytotoxin assay negative patients may be asymptomatic carriers[9,12]. In the present study just two patients tested negative with stool cytotoxin assay were positive with toxigenic culture.

Comparison between stool cytotoxin assay results with culture method (Table 4) showed that 45 fecal samples that tested positive with culture were negative by stool cytotoxin assay and 7 samples that tested negative with culture were positive by stool cytotoxin assay. Culture on selective medium is very sensitive but lacks specificity because of possible carriage of non-toxigenic isolates. Moreover, it requires a 40-48 h incubation time and is therefore a relatively slow technique. In this study, the culture method had low specificity, since by this method both toxigenic and nontoxigenic strains of *C. difficile* were identified.

Clostridium difficile is the major cause of antibiotic associated diarrhea as well as nosocomial diarrhea[1,13]. The use of the appropriate antibiotic therapy is crucial to prevent the progression of *C. difficile* pathogenesis[9]. Thus, the rapid diagnosis of this pathogen is decisive in allowing clinicians to prescribe the appropriate therapy[9]. Various laboratory methods may be used to detect the presence of *C. difficile* or its related toxins[11,14]. The stool cytotoxin assay is considered the gold standard for the biological diagnosis of disease associated with *C. difficile*, since it is specific and highly sensitive[9,15]. However, this assay also has some drawbacks. It is time consuming, as it requires an incubation period of 24 to 48 h, the facilities required are relatively elaborated (cell culture), requires cell culture expertise and there is the possibility of atypical cytotoxic effects, which then need to be neutralized.

Diagnosis by culture is also limited by the detection of both nontoxigenic and toxigenic strains of *C. difficile*. The requirement for a 48 to 72 h delay before obtaining a result if confirmation of strain toxigenicity is attempted is also a significant limiting factor[10,16]. In effort to overcome these difficulties, many enzyme Immunoassays have become commercially available during the last 15 years[11,15]. Thus, many hospitals now use a rapid *C. difficile* Tox A or Tox A/B immunoassay to diagnose CDAD, despite sensitivities of the rapid enzyme immunoassays being inferior to that of stool cytotoxin assay[8,17]. These tests offer significant advantages over stool toxin assay and may therefore be considered for use in clinical microbiology laboratories, particularly those that do not have tissue culture facilities. Additionally, any of these tests have the potential to be used in conjunction with toxigenic culture to attain maximal sensitivity. The rapid reporting of negative results provided by enzyme immunoassay should reduce the need for private rooms and contact isolation precautions and prevent the occasional delays in hospital discharges incurred while waiting for *C. difficile* test results. Contact precautions require the use of a private room and the donning of a gown and gloves upon entering a patient's room[18]. A disposable gown and gloves cost an estimated 0.90 USD. At an average of 10 patient contacts per day, this result in a cost of 18.00 USD in USA for 48 h until a negative stool cytotoxin assay result is reported[15]. Furthermore, the rapid negative test result would obviate most of the empiric treatment for CDAD, which now occurs with the delay in stool cytotoxin assay results. While the cost of oral metronidazole treatment is minimal but, exposure to voncomycin has been identified a risk factor for the vancomycin-resistant enterococci[15,18].

Present study suggests that the Tech Lab Tox A/B enzyme immunoassays may represent a satisfactory approach to routine testing for evidence of CDAD. In addition, the Tox A/B immunoassay test performs assays for both *C. difficile* toxins A and B: while strains producing only toxin B do not appear to be common, they have been implicated in human disease[9]. Thus, Tox A/B immunoassay represents a helpful and practical test, which can be used on stools for routine investigation of antibiotic-associated diarrhea.

In conclusion rapid and sensitive diagnostic tests for laboratory confirmation of CDAD are important in the current health care environment in order to initiate specific antibiotic treatment and to take adequate measures to control nosocomial spread.

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