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Original Article

Investigation of Various Tissue Culture Monolayers Sensitivity in Detection of *Clostridium difficile* Toxin

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Abstract

Backround: *Clostridium difficile* is the most common cause of nosocomial diarrhea. It is usually a consequence of antibiotic treatment, but sporadic cases can occur. The purpose of this study was to investigate five tissue culture monolayers sensitivity in detection of *C. difficile*-toxin.

Methods: A total of 402 stool samples from patients with nosocomial diarrhea hospitalized in three hospitals of Tehran University of Medical Sciences (TUMS) were collected. The samples were cultured on a selective cycloserine cefoxitin fructose agar (CCFA) and incubated in anaerobic conditions, at 37 °C for 4 days. Isolates were characterized to species level by conventional biochemical tests. Bacterial cytotoxicity was assayed on five tissue culture monolayers.

Results: Our findings show that of the total patients, 24 toxigenic C. *difficile* (6%) were isolated. All 24 *C. difficile* toxins showed cytotoxic effect at ≥ 1:10 dilution on Hela, Hep2, Vero, McCoy and Mdck cells after 16, 20, 24, 24 and 30 hours, respectively. *C. difficile* toxin showed cytotoxic effect at ≥ 1:100 dilutions only on Hela cell monolayer after 48 hours.

Conclusion: Hela cell monolayer may be a satisfactory substitute for the detection of *C. difficile* toxin in clinical specimens.

Keywords: *Patients, Diarrhea, Clostridium difficile, Toxin, CPe, Various tissue culture*

Introduction

In 1978 the association with *Clostridium difficile* and antibiotic induced pseudomembranous colitis (PMC) was established (1). Isolation rate of *C. difficile* varies from 90% in PMC to 20-25% in antibiotic associated diarrhea (AAD). Major risk factors include advanced age, duration of hospitalization, severity of underlying disease and exposure to antibiotics. The frequency of association is related to frequency of use, the route of administration and the impact of that antibiotic on the colonic microflora. This anaerobic bacterium has been identified as the leading cause of nosocomial infectious diarrhea and can be responsible for large out breaks.

Many strains of *C. difficile* produce two protein exotoxins, A and B, which are thought to be the primary causes of colonic mucosal injury and inflammation (2). Toxin A brings about primarily enterotoxic effects, while toxin B is primarily a cytotoxin. 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 a specific antigen, glutamate dehydrogenase (GDH), or toxins (A or B) in faecal samples (3). At present, the reference method is the stool cytotoxin assay, which reveals the presence of toxins in stool samples (3-5). The five tissue culture monolayers used for the initial determination of toxin detection sensitivity were Hela, Hep2, Vero, McCoy and Mdck.

The main goal of this study was to investigate of five tissue culture monolayers sensitivity in detection of *C. difficile*-toxin.

Materials and Methods

In this descriptive study from Dec 2004 to Sep 2006, 402 stool specimens of patients with nosocomial diarrhea hospitalized in three hospitals of Tehran University of Medical Sciences (Imam Khomeini Hospital, Shariati Hospital and Children Clinical Center) were collected. For isolation of *C.*

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difficile, selective cycloserine cefoxitin fructose agar (CCFA medium, Bio Merieux, France) was used. Plated were incubated under anaerobic condition (N2= 80%; CO2 10%; H2= 10%), for 48h at 37 °C. The isolates were identified as *C. difficile* by characteristic morphology, horse odour, green-yellow fluorescence under UV light and biochemical test (API20A; Bio Merieux, France) including lipase, lecithinase, catalase, H_2S_2 and indole production; gelatin, esculin and starch hydrolysis; and glucose, fructose, lactose, maltose and sucrose, fermentation. Bacterial cytotoxicity was assayed on Vero (African green monkey kidney) tissue culture monolayers. A filter-sterilized, 1:10 dilution of feces was used to inoculate Vero cell monolayers with and without neutralizing *C. difficile* antitoxin (Tech Lab) (6-8).

For investigation of bacterial toxin, 3-6 colonies of *C. difficile* were inoculated into Brain Heart Infusion (BHI) Broth (Oxoid) and incubated for 5-7 d at 37 °C. Then cultured BHI were centrifuged for 10 min at 2500 g. The supernatant was collected and passed through a 0.2 µm pore filter. Then serial dilutions were prepared (1:10, 1:100, 1:1000) in Eagle minimal essential medium (Gibco) supplemented with 2% fetal bovine serum.

Cell cultures were grown in polystyrene 150-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) with Eagle minimal essential medium (Gibco), to which were added 2 mM L-glutamine, 5 µg of gentamicin per ml, 25 U of my-costatin per ml, sufficient volume of 7.5% sodium bicarbonate to bring the solution to pH 7.2. and 10% heat inactivated fetal bovine serum. The cells were trypsinized by using 0.25% trypsin with 0.02% EDTA in phosphate buffered saline (pH 7.2; PBS). A viable cell count was performed with erythrosine B (0.08% in PBS), and preparation showing ≥90% viable cells were used. The cells were then diluted to 1.5×10^5 viable cells in growth medium for seeding. A 20-µl of each diluted filtrate, was added to four wells, and two of the wells received 20µl of a 1:50 PBS dilution of antitoxin (Tech lab, Inc., Blacksburg, Va) to neutralize the toxin. After 4, 8, 12, 24 and 48 h of incubation at 37 °C the cells (Hela, Hep2, Vero, McCoy, and Mdck) were examined for rounding. The endpoint of the titration was the last dilution showing ≥50% rounding of cells. Cells with toxin-negative filtrates or filtrates with antitoxin showed no rounding (3). The collected data and results of laboratory tests were analyzed by statistical package for social science (SPSS) and chi-square programme.

Results

Of total patients (208 males and 194 females, aged 3 yr- 65 yr), 24 toxigenic *C. difficile* (6%) were isolated. The highest and lowest rate of isolated toxigenic *C. difficile* was among age group of >60 yr old (11.9%) and 11-20 yr old (2.6%), respectively (Table1). Results of statistical analysis using Chi square test show significant differences between the rate of isolated toxigenic *C. difficile* and age group of patients ($P< 0.5$).

The results of testing are shown in Table 2. All 24 *C. difficile* toxins showed cytotoxic effect at ≥1:10 dilution on Hela, Hep2, Vero, McCoy and Mdck after 16, 20, 24, 24 and 30 h, respectively. *C. difficile* toxin showed cytotoxic effect at ≥1:100 dilution only on Hela cell monolayer after 48 h. In our study, tests were considered negative at ≥1:1000 dilution on all mentioned tissue culture monolayers.

Table 1: Patients with *C. difficile* nosocomial diarrhea based on sex and age

Age (year)	Male		Female		Total	
	%	n	%	n	%	n
<3 (n= 64)	4.7	3	1.9	1	6.3	4
3-10 (n= 46)	4.3	2	4.3	2	8.7	4
11-20 (n= 38)	2.6	1	-	-	2.6	1
21-30 (n= 42)	-	-	4.8	2	4.8	2
31-40 (n= 35)	2.9	1	2.9	1	5.7	2
41-50 (n= 36)	2.8	1	5.6	2	8.3	3
51-60 (n= 38)	5.8	2	2.6	1	7.9	3
> 60 (n= 42)	7.1	3	4.8	2	11.9	5
Total (n= 342)	3.8	13	3.2	11	7	24

Table 2: Rate of Intensity and speed effect of *C. difficile* toxin on various tissue culture monolayers

Tissue cultures	Hela			Vero			McCoy			Hep2			MDCK			Toxin and Antitoxin	Control	PBS
	Toxin dilutions			Toxin dilutions			Toxin dilutions			Toxin dilutions			Toxin dilutions					
Duration (h)	0/1	0/01	0/001	0/1	0/01	0/001	0/1	0/01	0/001	0/1	0/01	0/001	0/1	0/01	0/001			
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	+	±	–	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–
48	+	+	–	+	±	–	+	±	–	+	±	–	–	±	+	–	–	–

Discussion

C. difficile is a major cause of antibiotic associated diarrhea as well as nosocomial diarrhea (9-11). Applying an appropriate antibiotic therapy is crucial to prevent the progress of *C. difficile* pathogenesis (12). Acquisition of *C. difficile* occurs primarily in the hospital setting, where the organism has been cultured from bed rails, floors, windowsills, and toilets, as well as the hands of hospital workers who provide care for patients with *C. difficile* infection (4, 6, 13, 14). The organism can persist in hospital rooms for up to 40 d after infected patients have been discharged (1). The rate of *C. difficile* acquisition is estimated to be 13% in patients with hospital stays of up to two weeks and 50% in those with hospital stay longer than four weeks (15).

In spite of the growing number of studies on CDAD in Western countries, studies on CDAD are limited in the Middle East, especially in Iran, where information on the incidence of *C. difficile* carriage and CDAD is lacking. This is partly due to inertia in anaerobic bacteriology prompted, until recently, by lack of expertise, technology and facilities for culturing anaerobic pathogens.

Rapid diagnosis of this pathogen is decisive in allowing clinicians to prescribe the appropriate therapy (2). Various laboratory methods may be used to detect the presence of *C. difficile* or its related toxins (4-6, 13). The Stool cytotoxin assay (tissue culture cytotoxicity assay) is considered the gold standard for the biological diagnosis of the disease associated with *C. difficile*, since it is specific and highly sensitive (3-6).

The purpose of our study was to investigate the five tissue cultures (Hela, Hep2, Vero, McCoy and Mdck) sensitivity to *C. difficile* toxin. Maniar AC, et al (1984) reported that Mc Coy cell Monolayer is substitute for the detection of *C. difficile* toxins in clinical specimens (16). Maniar et al. reported, that comparative studies on several cell lines (AGMK, MRC-5, RMK, Vero) proved only Vero could be used as equivalent to McCoy in detection of *C. difficile* toxin (17). Draganov et al. reported that McCoy cell lines were widely applied in diagnostics and culture of various microorganisms with medical importance (18). Results of our study showed that, HeLa cell line was more suitable than others were. Hela cells showed cytotoxic effect within 16 h. While, Hep2, Vero, McCoy and Mdck cells required up to 20 h to show toxic effect.

In conclusion, Hela cells give results comparable to those of others and offer a practical alternative for the rapid detection of *C. difficile* cytotoxin.

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The authors declare that they have no Conflict of Interests.

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