

Prevalence of *Clostridium Difficile*- Associated Diarrhea in Hospitalized Patients with Nosocomial Diarrhea

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Abstract

Clostridium difficile is a frequently identified cause of nosocomial gastrointestinal disease. It has been proved to be a causative agent in antibiotic-associated diarrhea, antibiotic-associated colitis, and pseudomembranous colitis. This study was aimed to determine the prevalence of *C.difficile*- associated diarrhea in hospitalized patients with nosocomial diarrhea. The 942 hospitalized patients stool samples with nosocomial diarrhea were collected at three hospitals in Tehran from Dec 2002 to Feb 2004. All the stool samples were cultured and in 97 (prevalence: 10.9%) samples grew *C.difficile* that 57 (prevalence: 6.1%) isolates were toxigenic by cytotoxicity assay and so 57 patients had *C.difficile*- associated diarrhea. Results of statistical analysis showed significant difference between the rate of *C.difficile* associated diarrhea and the patients ages ($P<0.05$).

Keywords: Toxigenic *Clostridium difficile*, Antibiotic-associated diarrhea, Nosocomial diarrhea, Cytotoxicity assay, Iran

Introduction

Clostridium difficile-associated diarrhea (CDAD) is a very common nosocomial infection. (1). This is probably due to greater awareness of the disease among physicians, improved diagnostic methods, and a truly increased incidence of CDAD. The clinical expression of *C.difficile* infection ranges from asymptomatic infection to diarrhea (the most frequent clinical symptom) to pseudomembranous colitis, severe abdominal pain, toxic megacolon, bowel perforation, and death. *C.difficile* is responsible for 15-25% of all cases of antibiotic associated diarrhea and colitis and for more than 95% of cases of pseudomembranous colitis (2- 6).

C.difficile produces two toxins, toxin A (enterotoxin) and toxin B (cytotoxin), which are thought to be the primary causes of colonic mucosal injury and inflammation (7). Strains of *C. difficile* that do not produce the toxins are not pathogenic. There is not a single rapid laboratory test, both sensitive and specific for diagnosing CDAD. The cell cytotoxin assay (the "gold standard" method) is the most specific test, and although stool culture is the most sensitive test, it does not distinguish toxigenic from non-toxigenic strains, and both require at least 2 d to yield results (8, 9). Culture is the only diagnostic method that permits epidemiological analysis, but the production of toxins by

the isolate should be demonstrated.

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

In this study, the prevalence of CDAD in hospitalized patients with nosocomial diarrhea was examined.

Materials and Methods

Study site and stool specimens During the study period (from December 2002 to Feb 2004), 942 faecal samples were screened for presence of *C.difficile* and its toxins. These 942 samples, from three Tehran University hospitals (Emam Khomeini, Shariatei, and Children's Medical Center) in Tehran, Iran, fell into two groups, the first, consisted of samples for which the clinicians had specifically requested examination for *C.difficile* toxin (160 samples) and the second, consisted of 782 stool samples, which were selected by laboratory criteria. All the stool samples were tested by two techniques: stool cytotoxin assay for the presence of toxins in stool, and culture for the organism with follow-up toxin testing (toxigenic culture). 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 receiving samples) or stored at -20° C until they were tested.

Stool Culture and isolation For culture, a 1mL aliquot of stool was mixed thoroughly with approximately equal volume of Brain Heart Infusion Broth and mixed with twice the volume of ethanol 95%. After held at room temperature for 45 min, the sample was inocu-

lated on cycloserine-cefoxitin-fructose agar (CCFA) for isolation of *C.difficile*. This medium was supplemented by 10% horse serum, cycloserine (250 mg/l) and cefoxitin (8 mg/l). Plates were incubated in an anaerobic chamber for 48 h at 37°C (9, 10). Colonies that were suspected of being *C.difficile* on the basis of characteristic colony morphology, odor, and Gram stain morphology, were identified by standard procedures (8). Toxins were detected from *C.difficile* strains by toxigenic culture (9).

Stool toxin assay (Cytotoxin assay) A filter-sterilized, 1:10 dilution of feces was used to inoculate Vero cell monolayers with and without neutralizing *C.difficile* antitoxin (Tech Lab). Tissue cultures were examined for 24 and 48 h. Characteristic cytopathic effect (CPE) neutralized by antitoxin was interpreted as a positive result. Where a cytopathic effect was observed with a 1:10 dilution of feces and neutralized by antitoxin, the assay was repeated using higher dilutions (1:40 and 1:100) of feces.

Results

In this study *C.difficile* was isolated from 97 (prevalence: 10.3%) patients of which 40 and 57 isolates were nontoxigenic and toxigenic by cytotoxin assay and toxigenic culture, respectively. Since, the stool toxin assay (tissue culture cytotoxicity assay) is considered the gold standard for the biological diagnosis of CDAD (11, 12), our study showed that 57 patients (prevalence: 6.1%) with a positive result by cytotoxicity assay had CDAD.

Thirty six out of 57 patients with CDAD were male, so the infection rate in men and women was 7% and 5%, respectively, but there was no significant difference between men and women ($P>0.05$). Frequency of patients with CDAD and their genders is shown in Table 1.

The infection rate has got a significant difference in the various age groups ($P<0.05$). Frequency of the patients with CDAD and their ages is shown in Table 2.

The greatest parts of infections were detected at

Children’s Medical Center. Frequency of the patients with CDAD at the three hospitals is shown in Table 3.

One or more antibiotics had been taken during the preceding 4 weeks in patients with CDAD were as the follows: ampicillin, amoxicillin or third-generation cephalosporins 32%,aminoglycosides 20%, tetracyclines 15%, quinolones and fluoroquinolones 13%, clindamycin 10% and other agents 10%. The followings are the antibiotics which had been prescribed in other patients: fluoroquinolones 22%, tetracyclines 14% aminoglycosides 11%, penicillins and cephalosporins 1%, other agents 9% and without any history of antibiotic usage during the preceding 4 weeks 33%.

Among hospitalized patients with CDAD, the rate of infection rises rapidly from 8% for patients hospitalized 1-2 weeks to 47% for patients >4 weeks.

Table 1: Gender of patients with *Clostridium difficile* associated- diarrhea in this study

Results	Positive		Negative		Total	
	No	%	No	%	No	%
Male	36	6.9	489	93.1	525	55.7
Female	21	6	396	94	417	44.3
Total	57	6.1	885	93.9	942	100

Table 2: Distribution of age in patients with *Clostridium difficile* associated- diarrhea in this study

Results	Positive		Negative		Total	
	No	%	No	%	No	%
1-6 months	10	7.4	125	92.6	135	14.4
7-11months	5	5.1	92	94.9	97	10.3
1-3 yr	12	10.3	104	89.7	116	12.3
4-5 yr	7	7.2	90	92.8	97	10.3
6-10 yr	4	5.1	74	94.9	78	8.3
11-20 yr	5	4.9	98	95.1	103	10.9
21-30 yr	5	4.2	113	95.8	118	12.5
31-40 yr	3	3.4	84	96.6	87	9.2
> 40 yr	6	5.4	105	94.6	111	11.8
Total	57	6.1	885	93.9	942	100

Table 3: Prevalence of *Clostridium difficile* associated-diarrhea in this study

Results	Positive		Negative		Total	
	No	%	No	%	No	%
Children’s Medical Center	41	7.2	526	92.8	567	60.2
Emam Khomeini	12	5.1	223	94.9	235	24.9
Shariati	4	2.9	136	97.1	140	14.9
Total	57	6.1	885	93.9	942	100

Discussion

Clostridium spp. are part of intestinal indigenous microbiota in some people and they can produce several endogenous infections (11). *C.difficile* is a major cause of antibiotic associated diarrhea as well as nosocomial diarrhea (10, 12, 13). Rapid diagnosis of this pathogen is decisive in allowing clinicians to prescribe the appropriate therapy and to take adequate measures to control nosocomial spread (10).

Various laboratory methods may be used to detect the presence of *C.difficile* or its related toxins (9, 14-16). The stool toxin 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 (8, 9, 16). Also the procedure combining cytotoxin assay and toxigenic culture allows the highest sensitivity and specificity to be reached but, these two assays also have some drawbacks. Cytotoxin assay 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 con-

firmation of strain toxigenicity is attempted, is also a significant limiting factor (16). Also by stool toxin assay and or toxigenic culture, we could not detect strains which produce just one toxin (strains with toxin A⁻ B⁺), because both of toxins are cytotoxic and have a potent on the cytoskeletal or microfilament structure of mammalian tissue culture cell lines and so both toxins are simultaneously detected by these methods (17, 18).

In effort to overcome these difficulties, many enzyme immunoassays have become commercially available during the last 15 years (9, 14). 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 (18).

We undertook this study to determine the prevalence of CDAD in patients with nosocomial diarrhea. Toxigenic *C.difficile* was isolated from 6.1% of the patients with nosocomial diarrhea. Risk factors for *C.difficile* colonization and toxin production have been described previously. Antibiotic such as third generation cephalosporins are accepted as the main risk factors, and antibiotic restriction is the most effective control measure, so the increase in widespread and indiscriminate use of antibiotics in Iran raises the concern that CDAD may become a significant cause of hospital-acquired diarrhea in the country (19).

CCFA medium is recommended for the isolation of *C.difficile*; however, other species of clostridia also grow and produce a characteristic yellow fluorescence under UV light (20, 21). *C.difficile* was isolated from 10.9% of the patients. According to a previous study, *C.difficile* was isolated from 4.9% of Turkish patients with diarrhea (22).

All the 57 hospitalized patients with toxigenic *C.difficile*-positive stool samples were taking antibiotics at the time of the sampling. It is believed that some factors such as immunological alterations, age, nutritional conditions, genetic factors, pathologies or antimicrobial therapy,

can also interfere on the *C.difficile* isolation (23).

The recovery of *C.difficile* in patients with diarrhea could represent a small fraction of their intestinal microbiota, or be a fecal-oral contamination, but isolation of toxigenic *C.difficile* showed that patient was suffering from *C.difficile*-associated infection (24). In this study, 57 *C.difficile* isolates were toxigenic by stool toxin assay and toxigenic culture on Vero cell line and cytopathic effect was neutralized by specific *C.difficile* antitoxin.

These results also suggest the need of more studies to evaluate the role of *C.difficile* in diarrhoeal processes, which could provide a better understanding of such infections, as ecological and pathogenic terms.

Although little data has been published on the prevalence of *C.difficile* infection or the epidemiology of *C.difficile*- associated diarrhea in Iran, the isolation of toxigenic *C.difficile* from hospitalized patients showed that this pathogen was responsible for some cases of diarrhea with unknown origins and validates our efforts to establish its significance and conduct epidemiological studies in Iran.

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