Iranian Journal of Microbiology

Volume 13 Number 6 (December 2021) 793-800

ORIGINAL ARTICLE

Toxin gene profiles and antimicrobial resistance of *Clostridioides difficile* infection: a single tertiary care center study in Iran

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Received: April 2021**, Accepted:** August 2021

ABSTRACT

Background and Objectives: Due to the reduced susceptibility of clinical *Clostridioides difficile* strains in hospitals to various antimicrobial agents, the importance of antimicrobial susceptibility testing (ASTs) has increased. This study aimed to investigate the toxin gene profiles and the antimicrobial resistance of *C. difficile* isolated from hospitalized patients suspected of having *Clostridioides difficile* infection (CDI) in Tehran, Iran.

Materials and Methods: The stool samples were obtained from a hospitalized patients. The samples were shocked by alcohol and the patients cultured on cycloserine-cefoxitin-fructose agar in anaerobic Conditions. Toxin assay was performed for detection of toxinogenic isolates. An antibiotic susceptibility test was done. Furthermore, their genome was extracted for PCR to confirm *C. difficile* and detect toxin gene profile.

Results: Toxigenic *C. difficile* were identified in 21 of the 185 stool samples (11.3%). PCR detected seven toxin gene profiles; the highest prevalence was related to $tcdA^+B^+$, $cdtA^+B^-$ toxin gene profile (57.1%). There were 14.3% and 28.6% resistant rates of the isolates towards vancomycin and metronidazole with the toxin gene profiles; $tcdA^+B^+$, $cdtA^+B^+$; and $tcdA^+B^-$, $cdtA^-B^+$. All resistant isolates to moxifloxacin, clindamycin, and tetracycline were belonged to the toxin gene profiles; $tcdA^+B^+$, $cdtA^+B^+$; $tcdA^+B^+$, $cdtA^+B^-$, and $tcdA^-B^+$, $cdtA+B^-$.

Conclusion: Relative high resistance was detected towards metronidazole and vancomycin, although, still have acceptable activity for CDI treatment. However, a proper plan for the use of antibiotics and more regular screening of *C. difficile* antibiotic resistance seems necessary.

Keywords: *Clostridioeides difficile*; Multiplex-polymerase chain reaction; Toxin gene profiles; Antimicrobial resistance; Iran

INTRODUCTION

Clostridioides difficile (previously clostridium) is an anaerobic Gram-positive, spore-forming, toxin-producing bacterium, and it is an important nosocomial pathogen responsible for antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (1). Toxin A (enterotoxin, 308 kDa) and toxin B (cytotoxin, 270 kDa) are the major virulence factors and are located in pathogenicity locus (PaLoc) (1). Some *C. difficile* species can also produce binary toxins A and B encoded by the *cdtA* and *cdtB* genes, respectively (1, 2). Enzyme immunoassays (EIAs) for toxins A or B or both, EIAs for glutamate dehydrogenase (GDH), cell cytotoxin neutralization assay (CCNA), toxigenic culture (TC), Immunochromogenic assay,

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and PCR-based methods are laboratory tests used for diagnosis of *C. difficile* infection (CDI) (3). Chemotherapy is the most common risk factor for CDI (2). Almost all commonly used antibiotics can cause *C. difficile*-associated diarrhea (CDAD) (2). A dramatic increase in incidence and morbidity of CDI has been reported in many countries, often associated with hypervirulent strains (4). Increasing resistance and developing novel resistance mechanisms to important clinical antibiotics are growing concerns (2). Genetic analyses and antibiotic susceptibility testings (ASTs) have been used to characterize the clones isolated from outbreaks and severe infections (4). AST for *C. difficile* is complex, labor-intensive, and too expensive for routine clinical laboratory practice. However, ASTs can be pretty substantial, especially for the detection of hypervirulent strains (5). These strains often are associated with the consumption of fluoroquinolones and produce the binary toxin. Additionally, eradicating emerging *C. difficile* resistant isolates in hospitals can help evaluate the effectiveness of infection control practices. This study aimed to investigate the toxin gene profiles and antimicrobial resistance of *C. difficile* clinical isolates in hospitalized patients suspected of having CDI in Tehran, Iran.

MATERIALS AND METHODS

Specimen collection and study design. From April 15, 2016, until June 27, 2018, a total of 185 unformed (n: 61) and liquid (n: 124) stools specimens were collected from consecutive hospitalized patients suspected of having CDI (79 females and 106 males with an age range of 51 to 85 years; mean, 62 ± 15 years) at Firouzabadi hospital (single tertiary care center, 212 beds) in the south of Tehran, Iran. The included criteria were diarrhea symptoms, age over 50 years old, long-stay hospitalization (more than three days), taking antibiotics during the hospitalization, or having operations. The diarrhea was diagnosed as watery or loose, bloody or mucoid stool which has been passed at least three times a day. They completed a questionnaire containing different clinical and personal data, including clinical symptoms, use of antibiotics, and underlying conditions.This project was approved by the Iran University Human Ethics committee (Ethical code: IR.IUMS.FMD.REC 1396.33070).

Stool specimens were transported to the laboratory and processed immediately. They were directly cultured on CCFA agar Plate (CCFA: cycloserine-cefoxitin-fructose agar) (HiMedia, India) supplemented with 10% defibrinated sheep blood and selective components (8 µg/mLcefoxitin and 250 µg/mL cycloserine) following alcohol shock (6). The plates were incubated anaerobically (Whitley Jar Gassing System, UK) at 37°C for up to 5 days and examined daily for growth. Typical colonies phenotype was yellow circular or gray-white with raised centers and irregular filamentous or opaque edges, Gram stain, and positive Pro-disk test (for detection of the enzyme, L-proline aminopeptidase in *C. difficile* and yeast) performed for all suspected isolates (7).

Molecular determinants of toxin genes profile in *C. difficile* isolates. According to the manufacturer's protocol, total microbial DNA was extracted from bacteria on CCFA medium by FavorPrepTM Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp, Taiwan). DNA purity, quality, and quantity were measured by absorbance spectrophotometry (Nanodrop-1000; NanoDrop Technologies, Wilmington, DE. USA). Whole extracted DNAs were immediately stored at -20°C. Specific primers were used to detect glutamate dehydrogenase (*gluD*) and 16S rDNA that targets *C. difficile* housekeeping gene. Furthermore, the isolates were tested by 5-plex PCR for detection of toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtA/cdtB*) genes. Primers sequences are shown in Table 1 (8, 9). PCR reactions were run on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA). Gels were electrophoresed under standard conditions on 1.5% agarose and stained with EcoDye™ DNA Staining Solution (BIOFACT, South Korea). In parallel, for *in vitro* toxicity assay of *C. difficile* isolates, 10^4 Vero cells (C101, NCBI, Pasteur Institute of Iran, Tehran, Iran) were incubated with broth culture supernatant of various isolates for 48 h at 35°C in 5% CO_2 and then examined using an inverted microscope after 24 and 48 h for cytopathic effect (CPE) (7).

Antimicrobial susceptibility testing (AST). The agar dilution method was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines for vancomycin, metronidazole, moxifloxacin, clindamycin, and tetracycline (Sigma-Aldrich, St. Louis, Mo) (10). The antimicrobial working ranges expressed in MIC values (µg/mL) were the following: metronidazole 0.016-64; vanco-

Table 1. 5-plex PCR primers are from (9), except for two degenerate nucleotides (R and Y) added at position 11 and 14 of reverse primer of *tcdB*, respectively also for the forward primer of *cdtA* two degenerate nucleotides (R and Y) added at position 6 and 9.

PCR primers	Gene target	Sequence (5'–3')	Final Primer concentration (µM)	Amplicon size (bp)
5-plex PCR	*tcdA*	F-GCATGATAAGGCAACTTCAGTGGTA	0.6	629
		R-AGTTCCTCCTGCTCCATCAAATG	0.6	
	tcdB	F-CCAAARTGGAGTGTTACAAACAGGTG	0.4	410
		R-GCATTTCTCCRTTYTCAGCAAAGTA	0.4	
	cdtA	F-GGGAARCAYTATATTAAAGCAGAAGC	0.1	221
		R-CTGGGTTAGGATTATTTACTGGACCA	0.1	
	cdtB	F-TTGACCCAAAGTTGATGTCTGATTG	0.1	262
		R-CGGATCTCTTGCTTCAGTCTTTATAG	0.1	
C. difficile housekeeping genes	*16S rDNA*	F-GGAGGCAGCAGTGGGGAATA	0.05	1062
		R-TGACGGGCGGTGTGTACAAG	0.05	
	gluD	F- GTCTTGGATGGTTGATGAGTAC	0. 2	158
		R- TTCCTAATTTAGCAGCAGCTTC	0. 2	

mycin 0.016-8; moxifloxacin 0.064-32; clindamycin 0.256->256, and tetracycline 0.128-64. The inoculums was provided from BHI broth with suspensions of *C. difficile* from 24 h anaerobe blood agar plates. Turbidity was adjusted to an optical density equivalent to 0.5 McFarland standard (~1.5×10^8 CFU/ml). Brucella agar plates (HiMedia, India) supplemented with laked sheep blood (5% v/v), hemin (5 µg/mL), and vitamin K1 (1 µg/mL) were inoculated with 10 µl (10^5 CFU/spot) of the bacterial suspensions and incubated anaerobically (Whitley Jar Gassing System, UK) at 37°C for 48 h (11). All tests were performed in duplicates. *C. difficile* ATCC 700057 was used as a quality control strain for susceptibility testing. The MIC interpretative breakpoints of resistance, expressed in µg/mL, were: ≥ 32 for metronidazole, ≥ 16 for tetracycline, ≥ 8 for clindamycin, and moxifloxacin, according to CLSI recommendations (12). The MIC interpretive breakpoint for vancomycin was performed based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (MIC > 2 µg/mL) (13).

RESULTS

Toxin genes of *C. difficile* isolates. Based on our inclusion criteria, 185 stool samples were enrolled during the period of this study. All 185 stool samples were either unformed or liquid (14). Thirty stool samples (16.2%) were determined to be positive in the Pro-disk test, presence of *C. difficile* 16S rDNA, and housekeeping gene (*gluD*) by PCR (Fig. 1). As the PCR assay result, 21/30 *C. difficile* isolates were toxigenic (based on *tcdA/B* detection). They were also positive in toxigenic culture (TC) assay. Demographic and clinical characteristics of 21 patients with toxigenic *C. difficile* are mentioned in Table 2. The patients from which these isolates were recovered from distributed in different hospital wards, and 57.1% of

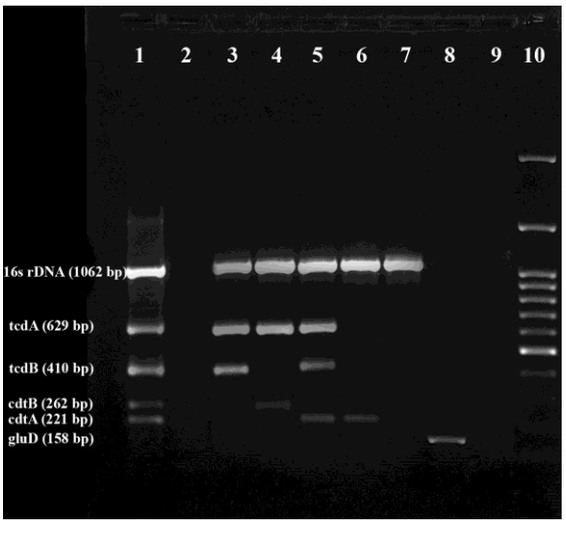

Fig. 1. 5-plex PCR Lan 1: $tcdA^+B^+$, $cdtA^+B^+$, Lan 2: negative control, Lan 3: $tcdA^+B^+$, $cdtA^-B^-$ Lan 4: $tcdA^+B^-$, $cdtA^-B^+$, Lan 5:3 $tcdA^+B^+$, $cdtA^+B^-$, Lan 6: $tcdA^-B^-$, $cdtA^+B^+$, Lan 7: $tcdA^-B^-$, $cdtA^-B^-$ *gluD* PCR Lan 8: positive *C. difficile* isolates, Lan 9: negative control, Lan 10: ladder 100kb.

Table 2. Demographic and clinical characteristics of 21 patients with *C. difficile* infection

Percentage	No. of patients	Characteristic
		Gender
(62)	13	Male
(38)	8	Female
		Age, years
(91)	19	51–68
(9)	2	> 68
		Hospital ward
(14.3)	3	Internal medicine
(23.8)	5	Intensive care unit
(28.6)	6	Infectious ward
(9.5)	2	Surgical ward
(19)	4	Gastroenterology
(4.8)	1	Other Laboratory parameters
(9.5)	2	Neutropenia
(52.4)	11	Leukocytosis
(14.3)	3	blood in stool
		Clinical parameters
(71.4)	15	Fever
(52.4)	11	Abdominal pain
		Exposure to Antibiotics
(52.4)	11	Penicillin
(57.14)	12	Cephalosporin
(28.6)	6	Clindamycin
(23.1)	5	Aminoglycoside
(23.1)	5	Fluoroquinolones
(14.3)	3	Metronidazole
(14.3)	3	Vancomycin
(28.6)	6	Other

them had taken beta-lactams antibiotics. The 5-plex PCR revealed six different toxin gene profiles (Fig. 2).

Antibiotic susceptibility of *C. difficile* isolates. Twenty-one toxigenic *C. difficile* isolates were tested for susceptibility to vancomycin, metronidazole, moxifloxacin, clindamycin, and tetracycline.

The toxin profiles and antimicrobial susceptibility of 21 toxigenic *C. difficile* isolates in this study were presented in Table 3. The vancomycin and metronidazole resistant isolates belonged to the three toxin gene profiles; $tcdA^+B^+$, $cdtA^\pm B^+$, and $tcdA^+B^-$, $cdtA^-B^+$. Isolates resistant to moxifloxacin, clindamycin, and tetracycline had toxin gene profiles; $tcdA^+B^+$, $cdtA^+B$, $tcdA^+B^+$, $cdtA^-B^-$ and $tcdA^+B^+$, $cdtA^+B^-$. All $tcdA^+B^+$, $cdtA^+B^+$ isolates were resistant to metronidazole, vancomycin, moxifloxacin, clindamycin, and tetracycline. Most patients (94.7%) hospitalized in infectious disease and ICU wards had bacteria with $tcdA^+$ and $tcdB^+$ phenotype. These patients had a history of antibiotics consumption, such as beta-lactams, aminoglycosides, and fluoroquinolones. The demographics data of the 21 patients with CDI were summarized in Table 1.

DISCUSSION

Clostridioides difficile infection is a growing concern for global public health (1). In this study, the prevalence of CDI in a single Iranian tertiary-care center from the 185 stool samples collected was found to be 11.3%. This observation is comparable with data from three Iranian center studies performed between 2016 and 2017 where *C. difficile* was detected in 14% (35/250) of patient stool samples and also relatively similar to other studies; 14.8%, Honda et al. 13.7% Hassan SA et al. (3), but lower than what was shown previously for the prevalence of CDI from other investigations; Moukhaiber et al. and Khoshdel et al. with 61.3 %, and 52%, respectively (15-18).

The incidence of $tcdA^-/tcdB^+$ *C. difficile* strains is extensively increasing and ranges from 3% to 92% worldwide (18). The prevalence of $tcdA^-B^+$ strains varies depending on the geographic region being studied. In a study conducted in Iran, the prevalence of $tcdA^-/tcdB^+$ strains was 8% (19). In Europe, 6.2% of *C. difficile* isolates were $tcdA^-/tcdB^+$ variant (20). However, no $tcdA^-/tcdB^+$ *C. difficile* strain was observed in our study. The role of binary toxins in disease is not well established. It may be associated with hypervirulent epidemic BI/NAP1/O27 strain, which increased CDI mortality (21).

The incidence of binary toxin in clinical *C. difficile* isolates varies from 1.6% to 34.6% (22). In our study, the binary toxin coding genes (*cdtA* and/or *cdtB*) were found in 52.4% of the 21 *tcdA* and *tcdB* positives isolate. From these, seven isolates were resistant to moxifloxacin. In contrast, lower binary toxin gene ($cdtA^+$ and/or $cdtB^+$) incidence rates were observed in Iran between 2016-2017, and a binary toxin gene prevalence of 10.5% was reported among 250 hospitalized patients from three hospitals (23).

Antimicrobial susceptibility is critically important when treating patients with CDI in hospitals as well as in community settings. In this study, five antimi-

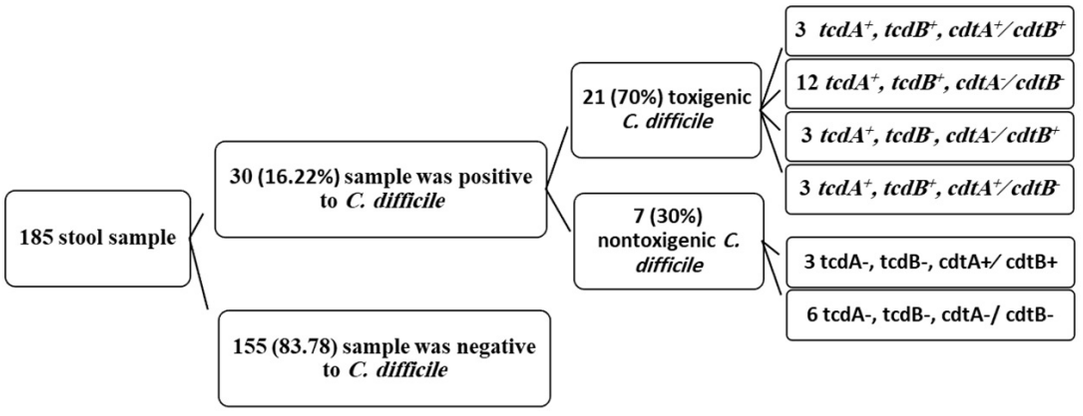

Fig. 2. Frequency of various toxin profilesof *C. difficile* in this study

Table 3. The toxin profiles and antimicrobial susceptibility of 21 toxigenic *C. difficile* isolates

Isolate ID	Toxin profiles						Antimicrobial susceptibility profiles									
	16s rRNA	*gluD*	*tcdA*	*tcdB*	*cdtA*	*cdtB*	Metronidazole		Vancomycin		Clindamycin		Moxifloxacin		Tetracycline	
							MIC (µg/mL)	R, I, S	MIC (µg/mL)	R, I, S	MIC (µg/mL)	R, I, S	MIC (µg/mL)	R, I, S	MIC (µg/mL)	R, I, S
1	+	+	+	+	+	+	32	R	4	R	32	R	32	R	32	R
2	+	+	+	+	+	+	32	R	4	R	8	R	16	R	16	R
3	+	+	+	+	+	+	64	R	8	R	32	R	8	R	16	R
4	+	+	+	+			0.256	S	0.08	S	2	S	0.064	S	8	I
5	+	+	+	+			0.016	S	0.016	S	2	S	0.512	S	4	S
6	+	+	+	+			0.08	S	0.256	S	32	R	8	R	1	S
7	+	+	+	+			0.256	S	0.256	S	4	I	0.128	S	0.512	S
8	+	+	+	+			0.016	S	0.016	S	4	I	0.064	S	1	S
9	+	+	+	+			0.08	S	2	S	2	R	4	S	8	I
10	+	+	+	+			2	S	0.08	S	2	R	0.512	S	4	S
11	+	+	+	+			4	S	0.08	S	32	R	1	S	4	S
12	+	+	+	+			0.08	S	0.256	S	8	R	2	S	2	S
13	+	+	+	+			32	R	0.016	S	32	R	0.512	S	2	S
14	+	+	+	+			32	R	0.08	S	8	R	0.512	S	16	R
15	+	+	+	+			2	S	0.256	S	32	R	8	R	16	R
16	+	+	+			+	0.256	S	0.08	S	0.256	S	0.512	S	2	S
17	+	+	+			+	0.08	S	0.256	S	1	S	2	S	4	S
18	+	+	+			+	32	R	0.256	S	2	S	2	S	4	S
19	+	+		+	+		0.256	S	0.08	S	128	R	0.512	S	32	R
20	+	+		+	+		0.256	S	0.256	S	8	R	8	R	32	R
21	+	+		+	+		0.016	S	0.256	S	> 256	R	8	R	64	R

S; sensitive, I; intermediate, R; resistance, MIC; Minimum Inhibitory Concentration

crobial agents, including the two antibiotics currently used as standard therapy for CDI, vancomycin, and metronidazole, were evaluated to determine MICs against the 21 toxigenic *C. difficile* isolates. Results indicated that 15 (71.4%) of the toxigenic *C. difficile* isolates were inhibited by 2 µg/mL of metronidazole, and 6 (28.6%) were resistant with MICs ≥ 32 µg/mL. A total of six patients were infected with metronidazole-resistant strains, and two strains were isolated from patients with pseudomembranous

colitis. The relatively high resistance of *C. difficile* to metronidazole exists in Iran. Resistance in Iran is higher than the global average (7, 8, 24). It may be attributed to the indiscriminate use of this drug in medicine (25). Also, the lack of completion of the treatment period leads to recurrent infection and increases the probability of *C. difficile* resistance to antibiotics (26). A gradual increase in metronidazole resistance has already been reported (7, 27). Interestingly, metronidazole is still the first-choice antimicrobial for treating mild to moderate CDI (26). Previous studies performed in Australia (28), Germany (29), and China (30) reported no metronidazole-resistance among *C. difficile* isolates, a finding not confirmed in the present study. Recently, a study performed from 2011 to 2017 in outpatients (n=45) and hospitalized patients (n=773) by Baghani et al. in Iran resulted in the isolation of highly-resistant phenotypes towards metronidazole (67.4%), moxifloxacin (78.3%), and tetracycline (82.6%) (8). Vancomycin is the first-line drug often used for moderate to severe CDI (26). There are currently no CLSI based breakpoints for vancomycin when testing *C. difficile.* According to the EUCAST vancomycin breakpoints, three strains (14.3%) had MICs > 2 µg/mL, classified as resistant. Snydman et al. and Tickler et al. had isolated *C. difficile* strains with vancomycin MICs of 4 µg/Ml (31, 32). Mutlu et al. in Scotland reported that vancomycin-resistant isolates with MICs of 4 µg/mL rapidly increased from 2.7% in 1999-2000 to 21.6% in 2005 (33). Resistance towards clindamycin was 57.14%. Incidences of *C. difficile* resistance to other antimicrobial drugs have also been reported. Various studies have reported a significant increase in the resistance rate to antimicrobial agents in Asian and European countries, such as clindamycin in Japan, Korea, and Iran, with 87.7%, 81%, and 89.3%, respectively (19, 34, 35). Regarding moxifloxacin, in the present study, MIC_{90} = 8 µg/mL, and three strains had MICs ≥ 16 µg/mL. Using CLSI breakpoints, 66.7% of the strains would be classified as susceptible and 33.3% resistant. This resistance rate is lower than that found in the United States, Europe, and Canada, with moxifloxacin-resistance rates of 36%, 39.9%, and 83%, respectively (2, 11, 36, 37). Around 14.3% of the tested isolates in this study showed intermediate susceptibility against tetracycline. *C. difficile* resistance to tetracycline varies among different countries from 2.4% to 41.67% (36). Five antibiotics of various classes were used in this study, and the presence of highly-resistant *C. difficile* was confirmed in Tehran (7, 8). The *C. difficile* MDR percentage was between 2.5% to 66% in various countries (8). The MIC_{50} and MIC_{90} values for tetracycline, and moxifloxacin in the $tcdA^+B^+$, $cdtA^+B^-$ strains were significantly higher than those for the $tcdA^+B^+$ strains: 4 and 5 in $tcdA^+B^+$, $cdtA^+B^-$ versus 2 and 2 in $tcdA^+B^+$, respectively. In the USA, Peng et al. (2017) (38) investigated antibiotic resistance and toxin production of 139 *C. difficile* isolates from patients diagnosed with CDI. They reported that there were 22 $tcdA^+B^+$, $cdtA^+B^+$ strains (95.65%, n = 23) showing resistance to more than 2 types of antibiotics were commonly associated with CDI, while in this study, there were 10 $tcdA^+B^+$, $cdtA^-B^-$ strains showing resistance to more than one type of antibiotics commonly associated with CDI.

In conclusion, despite the high relative resistance of *C. difficile* toward metronidazole and vancomycin, they still have acceptable activity for CDI treatment. Although to prevent increasing resistance, it is necessary to a proper plan for prescribing antibiotics and more regular monitoring of *C. difficile* antibiotic resistance.

ACKNOWLEDGEMENTS

We thank Mr. Lesan, Mr. Rahnama, and Mrs. Farahani from the microbiology laboratory of Firouzabadi hospitals, their help in isolating specimens. This study was financially supported by a research grant (97-01-30-33070) for an M.Sc. thesis at Iran University of Medical Sciences (Tehran, Iran), for which we are very grateful.

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