A multicenter-based study on epidemiology, antibiotic susceptibility and risk factors of toxigenic *Clostridium difficile* in hospitalized patients in southwestern Iran

Hadi Sedigh Ebrahim-Saraie[1], Hamid Heidari[1], Ali Amanati[2], Abdollah Bazargani[1], Seyed Alireza Taghavi[3], Iraj Nikokar[4], Kurosh Kalantar[5], Mohammad Motamedifar[1,6]

[1]Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;
[2]Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran;
[3]Gastroenterohepatology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran;
[4]Medical Biotechnology Research Center, Laboratory of Microbiology and Immunology of Infectious Diseases, Paramedicine Faculty, Guilan University of Medical Sciences, Rasht, Iran;
[5]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran;
[6]Shiraz HIV/AIDS Research Center, Institute of Health, Shiraz University of Medical Sciences, Shiraz, Iran

SUMMARY

Clostridium difficile (recently *Clostridioides difficile*) is a leading cause of hospital- and antimicrobial-associated diarrhea (AAD). The present study was carried out to investigate the prevalence of toxigenic *C. difficile*, antibiotic resistance and its associated risk factors in Iranian hospitalized patients. This cross-sectional study was conducted from October 2017 to June 2018 in three teaching hospitals in southwestern Iran. During this period, a total of 215 nonduplicated nosocomial AAD samples were collected from the hospitalized patients older than two years of age. Presumptive *C. difficile* isolates were identified by standard microbiologic methods and confirmed by specific PCR primers. The minimum inhibitory concentrations (MICs) were determined by the agar dilution method. PCR was carried out to determine the presence of toxin genes (*tcdA*, and *tcdB*). In all, from the 215 diarrheal samples, the frequency of *C. difficile* culture-positive samples was 21.4% (n = 46). Of the 46 *C. difficile* isolates, 43 carried both toxins, two isolates only had the *tcdB* gene, and one was negative for both toxins. Overall, all isolates of *C. difficile* were susceptible to metronidazole and vancomycin. The MIC50/MIC90 of metronidazole and vancomycin were 0.75/2 µg/mL, 0.25/0.75 µg/mL, respectively. The findings of this study show the prevalence of CDI in hospitalized patients in southwestern Iran, highlighting the importance of active surveillance of CDI in hospitals. Meanwhile, all of the tested isolates were susceptible to metronidazole and vancomycin, which encourages the use of these antibiotics as the drug of choice for initial treatment of CDI in our region.

Keywords: *Clostridium difficile*, toxigenic, metronidazole, vancomycin, nosocomial infections

INTRODUCTION

Nosocomial diarrhea is a major health concern due to increased morbidity, mortality and raise of hospitalization and healthcare cost in the developed and developing countries [1]. Amongst variety of pathogens including bacteria, parasites and viruses, *Clostridium difficile* (recently *Clostridioides difficile*) is the leading cause of hospital- and antimicrobial-associated diarrhea [2, 3]. *C. difficile* infection (CDI) is associated with a significant clinical burden ranging from mild cases include self-limiting antibiotic-associated diarrhea (AAD) to severe life-threatening pseudomembranous colitis (PMC), toxic megacolon, perforation of the

Corresponding author
Mohammad Motamedifar
E-mail: motamedm@yahoo.com;
motamedm@sums.ac.ir

colon, sepsis, and ultimately death [4]. CDI symptoms is mostly mediated by the production of at least one of the two major toxins, toxin A (TcdA, enterotoxin) and toxin B (TcdB, cytotoxin) that are encoded by the *tcdA* and *tcdB* genes, respectively [5]. Expression of these toxins causes pro-inflammatory and cytotoxic effects including disruption of the actin cytoskeleton and impairment of tight junctions in human intestinal epithelial cells, which are responsible for the clinical symptoms and pathogenesis of CDI [5].

CDI incidence is on the rise in term of frequency, severity, outbreaks, and recurrence, a consequence of indiscriminate and inappropriate use of broad-spectrum antibiotics [6]. However, the severity and outcome of CDI is influenced by a multiplicity of the host and environmental factors [7].

Metronidazole and vancomycin are the first drugs of choice for treating CDI, but there is a high rate of recurrence [8]. However, the standard antimicrobial therapy for CDI has changed due to reduced susceptibility or resistance to metronidazole or vancomycin [9]. Resistance to antibiotics in *C. difficile* varies widely between countries, but the emergence and spread of resistant strains is becoming an increasing public health concern [10]. Increased number of failed CDI treatment cases has been reported in different regions of the world, particularly in the northern part of Iran [10, 11].

Treating infections caused by anaerobic bacteria is mainly empirical and based on data derived from periodic surveillance studies [12]. However, due to diverse nature of antibiotic resistance patterns in periodic intervals, even in the same region, knowing the epidemiological profile of resistant strains can be helpful, when choosing an empirical therapy as well as to compare our situation with others. Owing to these facts and scarcity of studies in this field in Iran, the present study was carried out to investigate the prevalence of toxigenic *C. difficile*, antibiotic resistance and its associated risk factors in hospitalized patients in southwestern Iran.

PATIENTS AND METHODS

Study setting and design

This cross-sectional study was conducted from October 2017 to June 2018 in three teaching hospitals (Nemazee, Amir and Motahari hospitals) in Shiraz located in southwestern Iran. Nemazee Hospital with 1000 beds is a major tertiary care hospital in the southwestern Iran. The hospital has performed the first liver transplant and the first living related kidney transplantation in Iran. The hospitalization departments include internal, surgical, special and children's departments. Amir Oncology teaching hospital is the major cancer centers in Shiraz. This center provides specialized treatment for a wide range of cancers and blood disorders including surgery, chemotherapy and radiation therapy. During this period, a total of 215 nonduplicated nosocomial antibiotic-associated diarrhea samples were collected from the hospitalized patients. Patients younger than two years old were excluded. Diarrhea was defined as ≥3 loose and watery stools during a 24-hour period or fewer consecutive hours based on the definition of World Health Organization (WHO) [13]. Nosocomial antibiotic-associated diarrhea is defined as diarrhoic stool 72 hours after hospitalization which cannot be attributed to any other cause [14]. According to the European Centre for Disease Prevention and Control, an episode of CDI was defined as a patient with diarrhea whose stool takes the shape of a container, and it is positive for *C. difficile* toxin A and/or B without any other etiology [6]. Patients' clinical data with CDI were retrospectively reviewed to investigate the risk factors including demographic, admission date, purpose of admission, and antibiotic exposure prior the onset of CDI. This study was conducted in accordance with the declaration of Helsinki. It was also approved by the local Ethics Committee of Shiraz University of Medical Sciences (Approval No. IR.SUMS.REC.1396.S872).

Specimens and bacterial identification

Stool specimens were cultured on selective media cycloserine-cefoxitin fructose agar (CCFA) (MAST Diagnostic, UK) after the heat-shock procedure and incubated at 35°C for 48 hours in anaerobic conditions. Colonies suspected of being *C. difficile* were identified by Gram staining, typical morphology on agar plates as well as odor characteristic. Then, it was identified further by the amplification of triose phosphate isomerase (*tpi*) housekeeping gene as previously described [15]. The confirmed isolates were stored at -80°C for a long-term preservation.

Antimicrobial susceptibility testing
The minimum inhibitory concentrations (MICs) for metronidazole (MTZ) and vancomycin (VAN) were determined by the agar dilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) on Brucella agar plates supplemented with 1 mg/L vitamin K, 5 mg/L haemin and 5% defibrinated sheep red blood cells [16]. The range of MIC value used for metronidazole and vancomycin was 0.016 to 16 µg/mL. Resistance profiles of the isolates were determined according to the interpretative criteria recommended by the CLSI for metronidazole, while EUCAST breakpoints were applied for vancomycin [16, 17]. *C. difficile* ATCC 700057 was used as a quality control strain for antibacterial susceptibility testing.

DNA extraction and virulence genotyping
Genomic DNA was extracted from all isolates using the commercial DNA extraction kits (GeneAll, Korea) according to the manufacturer's instructions, subjected to polymerase chain reaction (PCR). Isolated genomic DNA was stored in Tris-EDTA buffer at -20°C until required for assays. PCR was carried out to determine the presence of *C. difficile* toxin genes (*tcdA* and *tcdB* genes) as previously described [15]. PCR amplifications for the studied genes were carried out in the following condition: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 45 sec, extension at 72°C for 50 sec, and final extension at 72°C for 5 min. PCR amplifications for the studied genes was carried out on a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). PCR products were separated by electrophoresis in 1.5% agarose gels with 1X TAE (Tris/Acetate/EDTA) buffer. DNA bands were observed by staining with safe stain load dye (Cinna Gen Co., Iran) and visualized under UV illumination.

Statistical analysis
The analysis was performed using SPSS™ software, version 21.0 (IBM Co., Armonk, NY, USA). The results are presented as descriptive statistics in terms of relative frequency. Values were expressed as the mean ± standard deviation (continuous variables) or percentages of the group (categorical variables).

RESULTS

A total of 215 diarrheal samples were collected from the hospitalized patients suspected of CDI during the aforementioned period in the mentioned hospitals. From the 215 diarrheal samples, the frequency of *C. difficile* culture-positive samples was 21.4% (n = 46). Moreover, all of the 46 isolates were further confirmed as *C. difficile* by the amplification of species-specific primers. Out of the total of 46 positive samples, 24 (52.2%) and 22 (47.8%) of the positive-cultures were from female and male samples, respectively. Patients with *C. difficile* had a median age of 29.5 years, ranging from 2 to 75 years old.
Of the 46 *C. difficile* isolates, 43 carried both *tcdA* and *tcdB* gene, two carried only *tcdB* gene, and one isolate was negative for both genes. The overall prevalence of CDI by estimating toxigenic isolates of *C. difficile* was 20.9% (45/215).
The full results of antibiotic susceptibility testing including MIC50 and MIC90 (MIC at which 50% and 90% of isolates were inhibited) of the tested isolates are shown in Table 1. Overall, all isolates of *C. difficile* were susceptible to metronidazole, and vancomycin. The MIC50/MIC90 of metronidazole and vancomycin were 0.75/2 µg/mL, 0.25/0.75 µg/mL, respectively.
Approximately, one to eight antibiotics (with an average 2.3 ± 1.5 antibiotics) had been used by patients prior the onset of CDI. Totally, 30.4% of patients received one, 34.8% received two and 21.7% received three types of antibiotics. The most commonly used antibiotic agent was carbapenem (60.9%) followed by fluoroquinolones (47.8%), and cephalosporins (43.5%). The most common underlying disease was ulcerative colitis 17.4% followed by gastroenteritis, liver transplantation, and acute lymphoblastic leukemia (ALL) each

Table 1 - Minimum inhibitory concentrations (MICs) and susceptibility profiles of *C. difficile* isolates toward the tested antimicrobial agents.

Antibiotics	MIC_{50} *(µg/mL)*	MIC_{90} *(µg/mL)*	*MIC mode (µg/mL)*	*MIC range (µg/mL)*	*Susceptible rate (%)*
Metronidazole	0.75	2	0.75	0.38-2	100
Vancomycin	0.25	0.75	0.19	0.125-1	100

with 10.9%. Out of 46 *C. difficile* positive cases, 37% were isolated from patients aged 2-17 years-old, 43.5% from 18-60 years-old, and 19.6% from patients older than 60 years-old. *C. difficile* colonization was mostly seen in patients with more than 3 months hospital stay (47.8%) followed by 1-3 months hospitalization (37%), and less than one month hospitalization (15.2%). The detailed results of demographic and clinical characteristics of colonized patients are presented in Table 2.

DISCUSSION

In recent years, the occurrence of CDI has increased significantly and has become a growing health concern, particularly in hospitals [18]. Hospital-associated CDI could be the consequence of disease evolution from colonized *C. difficile* or exposure to new strains transmitted by infected patients or the environment [19]. We found 21.4% of patients with nosocomial anti-

Table 2 - The full results of demographic and clinical characteristics of colonized patients.

No.	*Age*	*Gender*	*Underlying disease*	*History of Hospitalization*	*Antibiotic exposure*	*Toxin pattern*
1	45	M	NHL	>3 m	SXT-CIP	A+B+
2	5	F	Gastroenteritis	>3 m	CTX	A+B+
3	60	M	Diabetes mellitus	1-3 m	CIP-MER	A+B+
4	31	F	Liver transplantation	>3 m	CRO-IMI	A+B+
5	47	M	Kidney failure	<1 m	CRO-LEV-CIP	A+B+
6	26	F	Liver transplantation	>3 m	CRO	A+B+
7	40	M	Ulcerative colitis	>3 m	CRO-CIP	A+B+
8	62	M	Gastroenteritis	<1 m	CRO-CIP	A+B+
9	71	M	Ulcerative colitis	1-3 m	CIP-MER	A+B+
10	29	F	MDS	1-3 m	CAZ-CIP-MER	A+B+
11	3	F	Liver transplantation	>3 m	LZD-SXT-MER	A+B+
12	47	F	Autoimmune hepatitis	>3 m	CFM-CAZ-CRO-CIP-IMI-MER-LZD	A+B+
13	65	F	ALL	<1 m	CIP-MER	A+B+
14	36	F	Pneumonia	>3 m	AZT-SAM-CLX-CIP-SXT-ERY-LEV-MER	A+B+
15	6	M	Ulcerative colitis	>3 m	SXT-LEV-LZD-MER	A+B+
16	37	F	Pneumonia	1-3 m	SAM-LEV	A+B+
17	30	F	Gastroenteritis	1-3 m	CIP-SXT-MER	A+B+
18	28	M	Crohn's	1-3 m	CRO	A+B+
19	3	F	Ulcerative colitis	>3 m	CTX-MER	A+B+
20	21	M	Ulcerative colitis	<1 m	AZT	A+B+
21	2	M	Metabolic disorder (phenylalanine)	1-3 m	CFM-CRO	A+B+
22	37	M	Ulcerative colitis	>3 m	CIP	A+B+
23	45	F	AML	>3 m	CIP-CLI	A+B+
24	63	M	Kidney failure	1-3 m	MER	A+B+
25	2	F	Glaucoma	<1 m	CRO	A+B+
26	8	F	Liver transplantation	>3 m	CTX	A-B-
27	59	F	CLL	>3 m	IMI-MER	A+B+
28	69	F	Liver neoplasms	1-3 m	LEV-MER	A+B+

>>>

>>>

No.	Age	Gender	Underlying disease	History of Hospitalization	Antibiotic exposure	Toxin pattern
29	3	M	Liver transplantation	>3 m	MER-CTX-AMK	A+B+
30	56	M	Kidney failure	>3 m	CRO	A-B+
31	75	F	Multiple myeloma	>3 m	MER	A+B+
32	3	M	Metabolic disorder (phenylalanine)	>3 m	CRO	A+B+
33	21	M	Ulcerative colitis	>3 m	CIP-MER	A+B+
34	2	F	Gastroenteritis	1-3 m	MER-CTX-AMP	A+B+
35	73	F	Kidney failure	1-3 m	CRO-CAZ-AMK-CIP-MER	A+B+
36	63	M	Diabetes mellitus	>3 m	CIP-IMI-MER	A-B+
37	37	M	Ulcerative colitis	>3 m	CIP	A+B+
38	3	F	Gastroenteritis	>3 m	MER-CTX-SXT	A+B+
39	16	M	ALL	1-3 m	MER	A+B+
40	7	M	Optic Glioma	1-3 m	MER	A+B+
41	18	F	ALL	1-3 m	PTZ-TEC-CS	A+B+
42	2	M	ALL	1-3 m	CIP-SXT-MER-AMK	A+B+
43	10	F	Wilm's Tumor	1-3 m	AMK-MER	A+B+
44	10	M	Osteosarcoma	1-3 m	MER-SXT	A+B+
45	64	F	Colon Cancer	<1 m	MER-CRO-CAZ	A+B+
46	5	F	ALL	<1 m	MER-CIP-CLI-SXT	A+B+

F: Female; M: Male; NHL: Non-Hodgkin lymphoma; MDS: Myelodysplastic syndromes; ALL: Acute lymphoblastic leukemia; CLL: Chronic lymphocytic leukemia; AML: Acute myeloid leukemia; SXT: Co-trimoxazole; CIP: Ciprofloxacin; CTX: Cefotaxime; MER: Meropenem; CRO: Ceftriaxone; IMI: Imipenem; LEV: Levofloxacin; CAZ: Ceftazidime; LZD: Linezolid; CFM: Cefixime; AZT: Azithromycin; SAM: Ampicillin/sulbactam; CLX: Cloxacillin; ERY: Erythromycin; CLI: Clindamycin; AMK: Amikacin; PTZ: Piperacillin/Tazobactam; TEC: Teicoplanin; CS: Colistin.

biotic-associated diarrhea to be colonized with *C. difficile*, 97.8% of which were toxicogenic and considered as an episode of CDI. To date, several studies have described the prevalence of CDI in Iran and the Middle East amongst the hospitalized patients. Previously, in three separate studies conducted in the Northern region of Iran, the prevalence of CDI during 2011 to 2014 amongst hospitalized patients was reported 17.1% to 21.4% [11,20,21]. However, at the same period, there were some reports from southern regions of Iran indicating much lower rate of CDI in hospitalized patients [22, 23]. This inconsistency might be due to the differences in geographical distribution, infection control policies, detection methods, and the studied population. The prevalence of CDI among the hospitalized patients is also different throughout the world. In our neighboring countries, this rate was estimated at 21.7% in Saudi Arabia, 21% in Iraq, 8% in Qatar, and 5.6% in Turkey [24-27]. Meanwhile, a similar pattern was observed in Europe and United States surveillance for CDI among hospitalized patients with diarrhea [28-30].

All of our tested isolates were susceptible to metronidazole and vancomycin, which inhibited at a concentration of ≤2 and ≤1 µg/mL, respectively. The available reports on antimicrobial susceptibility of *C. difficile* toward metronidazole from the Northern region of Iran showed approximately 95% susceptibility to MIC50 of 0.5 and ≤8 µg/mL, and MIC90 of 2 and ≤8 µg/mL, respectively [11, 31]. Moreover, our findings are in line with previous reports from European and Asian countries that showed more than 95% susceptibility to metronidazole [6, 32-34], while lower susceptibility rate was observed in the United States [35]. Regarding the antimicrobial susceptibility of *C. difficile* toward vancomycin, the only Iranian report by Goudarzi et al. showed lower susceptibility rate in comparison to our findings (92% vs. 100%) [11]. Furthermore, their isolates showed higher MIC range compared to our isolates (MIC50/90 = 1/1 vs. 0.25/0.75 µg/mL). Meanwhile, our results

were in line with other studies in Asia and Europe where the resistance of isolates to vancomycin was rare or relatively low [6, 32-34].

Almost all antibiotics were reported to be associated with CDI; hence, exposure to antimicrobial agents considered as an important predisposing factor for CDI [36]. Carbapenem, the third-generation cephalosporins, and fluoroquinolones were the most frequently used antibiotics among CDI cases in our study. These findings were consistent with previous studies that showed broad-spectrum antibiotics with alterations in gut microbiota allowing the development of CDI [37].

A remarkable proportion of our CDI cases suffering from ulcerative colitis, previously it has been mentioned that inflammatory bowel disease (IBD) patient with colitis are at greatest risk of acquiring CDI [38, 39]. Several risk factors including preexisting injury in the intestinal mucosa, dysbiosis in gut microbiome, and use of immunosuppressive medications can predispose IBD patients to CDI [39].

In the present study, we found majority of CDI cases within patients less than 60 years-old. Reasons for such observation are not clearly apparent; however, severe underlying diseases in most young patients can be a reason for this observation. In addition, there are increasing reports of CDI amongst younger individuals, particularly in Asian countries [36, 40, 41].

The present study has some limitations that should be acknowledged. First, we performed a cross-sectional study, which was limited to patients with AAD and not all of the diarrhea patients were tested for *C. difficile*; hence the burden of infection would be expected to be different. Second, we only evaluated hospitalized patients; hence it cannot be stated with certainty that colonization had occured in hospitals or prior to admission.

Despite these limitations, findings of this multicenter based study showed the prevalence of CDI in hospitalized patients in southwestern Iran by highlighting the importance of active surveillance of CDI in hospitals. Meanwhile, all of the tested *C. difficile* isolates were susceptible to metronidazole and vancomycin. This result encourages the use of these antibiotics as the drug of choice for initial treatment of CDI in our region, due to lower cost and antibiotic resistance concerns. However, further epidemiological studies are necessary to determine the frequently found molecular types for surveillance of international spread of *C. difficile* strains.

Funding support

This study was supported by Shiraz University of Medical Sciences with grant No. 96-14947.

ACKNOWLEDGMENT

We would like to thank all the participants and staff at the studied hospital for their kind cooperation. This article was extracted from part of the PhD thesis by H. Sedigh Ebrahim-Saraie under the supervision of Dr. M. Motamedifar. The authors also wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

Conflict of interests

The authors declare that they have no competing interests.

REFERENCES

[1] Polage C.R., Solnick J.V., Cohen S.H. Nosocomial diarrhea: evaluation and treatment of causes other than *Clostridium difficile*. *Clin. Infect. Dis*. 55, 7, 982-989, 2012.

[2] Nasiri M.J., Goudarzi M., Hajikhani B., Ghazi M., Goudarzi H., Pouriran R. *Clostridioides* (*Clostridium*) *difficile* infection in hospitalized patients with antibiotic-associated diarrhea: A systematic review and meta-analysis. *Anaerobe* 50, 32-37, 2018.

[3] Heinlen L., Ballard J.D. *Clostridium difficile* infection. *Am. J. Med. Sci*. 340, 3, 247-252, 2010.

[4] Han S.H., Kim H., Lee K., et al. Epidemiology and clinical features of toxigenic culture-confirmed hospital-onset *Clostridium difficile* infection: a multicentre prospective study in tertiary hospitals of South Korea. *J. Med. Microbiol*. 63, 11, 1542-1551, 2014.

[5] Di Bella S., Ascenzi P., Siarakas S., Petrosillo N., di Masi A. *Clostridium difficile* Toxins A and B: Insights into Pathogenic Properties and Extraintestinal Effects. *Toxins (Basel)*. 8, 5, 2016.

[6] Jamal W.Y., Rotimi V.O. Surveillance of antibiotic resistance among hospital- and community-acquired toxigenic *Clostridium difficile* isolates over 5-year period in Kuwait. *PLoS One*. 11, 8, e0161411, 2016.

[7] Tenover F.C., Tickler I.A., Persing D.H. Antimicrobial-resistant strains of *Clostridium difficile* from North America. *Antimicrob. Agents. Chemother*. 56, 6, 2929-2932, 2012.

[8] Di X., Bai N., Zhang X., et al. A meta-analysis of metronidazole and vancomycin for the treatment of

Clostridium difficile infection, stratified by disease severity. *Braz. J. Infect. Dis*. 19, 4, 339-349, 2015.
[9] Spigaglia P. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection. *Ther. Adv. Infect. Dis*. 3, 1, 23-42, 2016.
[10] Peng Z., Jin D., Kim H.B., et al. Update on antimicrobial resistance in *Clostridium difficile*: resistance mechanisms and antimicrobial susceptibility testing. *J. Clin. Microbiol*. 55, 7, 1998-2008, 2017.
[11] Goudarzi M., Goudarzi H., Alebouyeh M., et al. Antimicrobial susceptibility of *Clostridium difficile* clinical isolates in Iran. *Iran. Red. Crescent. Med. J*. 15, 8, 704-711, 2013.
[12] Jamal W., Shahin M., Rotimi V.O. Surveillance and trends of antimicrobial resistance among clinical isolates of anaerobes in Kuwait hospitals from 2002 to 2007. *Anaerobe* 16, 1, 1-5, 2010.
[13] Jamal W., Pauline E., Rotimi V. A prospective study of community-associated *Clostridium difficile* infection in Kuwait: Epidemiology and ribotypes. *Anaerobe* 35, Pt B, 28-32, 2015.
[14] Kyne L., Hamel M.B., Polavaram R., Kelly C.P. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile. Clin. Infect. Dis*. 34, 3, 346-353, 2002.
[15] Lemee L., Dhalluin A., Testelin S., et al. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of *Clostridium difficile*. *J. Clin. Microbiol*. 42, 12, 5710-5714, 2004.
[16] Wayne P.A. Performance Standards for Antimicrobial Susceptibility Testing. *Clinical and Laboratory Standards Institute (CLSI)* 28th Informational Supplement., M100-S28, 2018.
[17] The European Committee on Antimicrobial Susceptibility Testing. *Breakpoint tables for interpretation of MICs and zone diameters, version 8, 2018*. http://www.eucast.org/clinical_breakpoints/.
[18] Depestel D.D., Aronoff D.M. Epidemiology of *Clostridium difficile* infection. *J. Pharm. Pract*. 26, 5, 464-475, 2013.
[19] Furuya-Kanamori L., Marquess J., Yakob L., et al. Asymptomatic *Clostridium difficile* colonization: epidemiology and clinical implications. *BMC Infect. Dis*. 15, 516, 2015.
[20] Alinejad F., Barati M., Satarzadeh Tabrisi M., Saberi M. Hospital acquired diarrhea in a burn center of Tehran. *Iran. J. Microbiol*. 7, 6, 310-314, 2015.
[21] Azimirad M., Krutova M., Nyc O., et al. Molecular typing of *Clostridium difficile* isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital. *Anaerobe* 47, 125-128, 2017.
[22] Rezazadeh Zarandi E., Mansouri S., Nakhaee N., Sarafzadeh F., Iranmanesh Z., Moradi M. Frequency of antibiotic associated diarrhea caused by *Clostridium difficile* among hospitalized patients in intensive care unit, Kerman, Iran. *Gastroenterol. Hepatol. Bed Bench* 10, 3, 229-234, 2017.
[23] Farshad S., Azami M., Pouladfar G., Ziyaeyan M., Aminshahidi M., Alborzi A. Prevalence and risk factors of *Clostridium difficile*-Associated diarrhea in Iranian hospitalized patients. *Ann. Trop. Med. Publ. Health*. 6, 5, 554-558, 2013.
[24] Sandokji A.M., Murshid K.R., El-Badry A.A., Al-Ali K.H., Shalaby S.A. Infectious nosocomial diarrhea in the surgical wards: role of parasites and microbes imply stool analysis. *J. Taibah. Univ. Med. Sci* 4, 1, 73-81, 2009.
[25] Alrifai S.B., Alsaadi A., Mahmood Y.A., Ali A.A., Al-Kaisi L.A. Prevalence and etiology of nosocomial diarrhoea in children <5 years in Tikrit teaching hospital. *East. Mediterr. Health J*. 15, 5, 1111-1118, 2009.
[26] Al-Thani A.A., Hamdi W.S., Al-Ansari N.A., Doiphode S.H., Wilson G.J. Polymerase chain reaction ribotyping of *Clostridium difficile* isolates in Qatar: a hospital-based study. *BMC Infect. Dis*. 14, 502, 2014.
[27] Deniz U., Ulger N., Aksu B., Karavus M., Soyletir G. Investigation of toxin genes of *Clostridium difficile* strains isolated from hospitalized patients with diarrhoea at Marmara University Hospital. *Mikrobiyol. Bul*. 45, 1, 1-10, 2011.
[28] Lessa F.C., Winston L.G., McDonald L.C. Burden of *Clostridium difficile* infection in the United States. *N. Engl. J. Med*. 372, 24, 2369-2370, 2015.
[29] Barbut F., Mastrantonio P., Delmee M., Brazier J., Kuijper E., Poxton I. Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin. Microbiol. Infect*. 13, 11, 1048-1057, 2007.
[30] Bauer M.P., Notermans D.W., van Benthem B.H., et al. *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* 377, 9759, 63-73, 2011.
[31] Shayganmehr F.S., Alebouyeh M., Azimirad M., Aslani M.M., Zali M.R. Association of tcdA+/tcdB+ *Clostridium difficile* genotype with emergence of multidrug-resistant strains conferring metronidazole resistant phenotype. *Iran. Biomed. J*. 19, 3, 143-148, 2015.
[32] Kunishima H., Chiba J., Saito M., Honda Y., Kaku M. Antimicrobial susceptibilities of *Clostridium difficile* isolated in Japan. *J. Infect. Chemother*. 19, 2, 360-362, 2013.
[33] Chow V.C.Y., Kwong T.N.Y., So E.W.M., et al. Surveillance of antibiotic resistance among common *Clostridium difficile* ribotypes in Hong Kong. *Sci. Rep*. 7, 1, 17218, 2017.
[34] Freeman J., Vernon J., Morris K., et al. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. *Clin. Microbiol. Infect*. 21, 3, 248.e249-248.e216, 2015.
[35] Snydman D.R., McDermott L.A., Jacobus N.V., et al. U.S.-Based National Sentinel Surveillance Study

for the Epidemiology of *Clostridium difficile*-Associated Diarrheal Isolates and Their Susceptibility to Fidaxomicin. *Antimicrob. Agents. Chemother*. 59, 10, 6437-6443, 2015.
[36] Khan F.Y., Abu-Khattab M., Anand D., et al. Epidemiological features of *Clostridium difficile* infection among inpatients at Hamad General Hospital in the state of Qatar, 2006-2009. *Travel. Med. Infect. Dis*. 10, 4, 179-185, 2012.
[37] Vincent C., Manges A.R. Antimicrobial use, human gut microbiota and *Clostridium difficile* colonization and infection. *Antibiotics (Basel)*. 4, 3, 230-253, 2015.
[38] Rezapour M., Galoosian A., Liu B., Bhuket T., Wong R.J. *Clostridium difficile* co-infection in inflammatory bowel disease is associated with significantly increased in-hospital mortality. *Eur. J. Gastroenterol. Hepatol*. 30, 9, 1041-1046, 2018.
[39] Binion D. *Clostridium difficile* infection and inflammatory bowel disease. *Gastroenterol. Hepatol. (N Y)* 12, 5, 334-337, 2016.
[40] Jalali M., Khorvash F., Warriner K., Weese J.S. *Clostridium difficile* infection in an Iranian hospital. *BMC Res. Notes*. 5, 159, 2012.
[41] Al-Tawfiq J.A., Abed M.S. *Clostridium difficile*-associated disease among patients in Dhahran, Saudi Arabia. *Travel. Med. Infect. Dis*. 8, 6, 373-376, 2010.