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Original Article

Antibiotic-Associated Diarrhea Mediated by Toxigenic *Clostridioides Difficile* Infection in Iran: A Clinical Survey by Multiplex Real-Time Polymerase Chain Reaction and Toxigenic Culture

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ABSTRACT

Background:
Clostridioides difficile is one of the important causes of hospital infections worldwide.
Regarding the importance of *Clostridioides difficile* infection (CDI) epidemics and limited available prevalence reports of CDI in Iran, the present investigation was done on the incidence of CDI in hospitalized patients from 2017-2018.
Our study outlined the requirements for CDI and followed up on the causative agent of antibiotic-associated diarrhea (AAD).

Materials and Methods:
We evaluated the CDI rates by multiplex real-time polymerase chain reaction (PCR) assay directly from inpatient fecal samples with a history of antibiotic therapy (2-8 weeks) and combined with anaerobic culture and toxicity assessments to isolate toxigenic and non-toxigenic types.
The results were analyzed through one-way analysis of variance (ANOVA), pairwise two-tailed correlation, and regression using SPSS software version 25.0 (IBM® SPSS® Statistics, USA).

Results:
Among 491 fecal samples, 49 (9.9%) toxigenic *C. difficile* were characterized by real-time PCR, while 40 were isolated by toxigenic culture and cytotoxicity assay. Toxin profiling showed 43 (9.7%) tcdA+/tcdB+ and 6 (1.4%) tcdB+.

Conclusion:
A considerable prevalence of CDI among patients with AAD was demonstrated, and causative organisms mostly produce both toxins A and B. Since *C. difficile* still has problematic treatment and is costly, rapid and early detection may help to curb *C. difficile* infection more efficaciously.

Keywords: Antibiotic-associated diarrhea; *Clostridioides difficile*; Real-time PCR; *tcd*A; *tcd*B

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INTRODUCTION

Clostridioides difficile (C. difficile) is an obligate anaerobic spore-forming gram-positive bacillus, which could be toxin-producing. *C. difficile* is the main cause of antibiotic-associated diarrhea (AAD), an ailment rapidly increasing in prevalence. *C. difficile* infection (CDI) risk factors are advanced age and multiple antibiotic therapies(1). Both antibiotic prophylaxis, which is recommended in sensitive inpatient groups and broad-spectrum antibiotic therapy prescribed in patients

suffering from an infection could cause a disturbance in normal gut microflora and increase the contracting C. difficile infection. Therefore, *C. difficile*-mediated infections are a major problem for infection control in hospitals, long-term healthcare systems, and nursing homes. If overgrown in the colon, *C. difficile* has the ability to produce enterotoxin or/and cytotoxin (A and B toxins, respectively) that cause severe mucosal damage(2,3). In the past two decades, *C. difficile* infection rates have increased dramatically among the high-risk population, and its detection and treatment are critical challenges for hospitals and healthcare systems. An accurate and timely diagnosis of the infection is critical for the effective treatment of *C. difficile* infections(4).

Anaerobic toxigenic culture of *C. difficile* on specific media, namely CCFA (Cycloserine-Cefoxitin-Fructose agar), is the sensitive and selective culture medium for isolation and characterization of the organism; however, this method cannot differentiate the pathogenic from non-pathogenic isolates. Many diagnostic methods routinely target single or both toxins in stool samples. Such methods are mostly based on cell cytotoxicity neutralization assay (CCNA) or enzyme immunoassays (EIA) and differ significantly in sensitivity, specificity, time, and cost. In addition, these tests should be combined with a screening of the glutamate dehydrogenase (GDH) gene as a complementary test, which is necessary for confirmation of C. difficile. Although GDH is a more sensitive target for confirming the *C. difficile* organisms, it cannot distinguish between toxigenic and non-toxigenic types (5). Non-toxigenic *C. difficile* does not cause infection, so it is important to differentiate toxigenic types while the presence of non-toxin-producing *C. difficile* does not indicate the infection (6). In this work, we targeted toxins A and B genes for the detection of *C. difficile* that are *tcd*A and *tcd*B, respectively.

Providing reliable data on the incidence and prevalence of CDI could help the better management of this long-lasting infectious challenge. We aimed to determine the prevalence of CDI among hospitalized patients in Iran by multiplex real-time PCR method and investigate if the data could be relied on for medical professionals as compared with toxigenic culture and cytotoxicity assays.

MATERIALA AND METHODS

Standard strains

Five clinical isolates of C. difficile were approved through PCR and sequencing of specific genes, including 16S rRNA, GDH gene (gluD) (7), *tcd*A, and *tcd*B genes (8). They all met the criteria in section 14.3 of Quality Assurance in Bacteriology and Immunology (third edition, WHO; 2012) and were used as standardized laboratory strains (designated as *C. difficile* MDS1-5).

DNA extraction

DNA extraction from bacterial cells was performed using the QIAamp® DNA Mini Kit (Qiagen). For fecal specimens, we used QIAamp® DNA Stool Mini Kit (Qiagen).

Study population and clinical specimens

The subject population comprised 491 patients who received antibiotic therapy and encountered diarrhea (AB+DR+) (9). Fresh stool samples were collected from the adult patients (<18 years old) hospitalized in the Gastroenterology, Bone Marrow Transplantation and Leukemia, Intensive care unit (ICU), and the other wards of the two hospitals (Tehran, Iran). The stool samples underwent diagnostic procedures on the same day they were collected or stored at 4°C for no more than 48h.

Cytotoxigenic culture tests

1.Anaerobic culture

One spike (~1g) from each stool sample was treated by alcoholic shock for 1 hour to inhibit the growth of other bacteria in feces. Another spike was transferred into the *Clostridioides difficile* Brucella broth (CDBB) and incubated for 1 hour to support the vegetative forms of *C. difficile* (10). Treated specimens were inoculated onto the cycloserine-cefoxitin fructose agar, enriched by vitamin K1 (1µg/mL) and hemin (5µg/mL) and incubated for 2-5 days at 37°C under anaerobic condition by using anaerobic jars (Merck) containing Anaerocult® A Gas Pack (Merck) (11). The colonies were then confirmed by microbiological characteristics such as colony morphology, gram stain, and horse manure odor (5). Isolated organisms were confirmed by PCR-tracing of glutamate dehydrogenase (GDH) gene (gluD) as

Table 1: Patient population groups

Patient population groups			
AB+DR+	**AB+DR-**	**AB-DR+**	**AB-DR-**
antibiotic therapy and diarrhea	antibiotic therapy without diarrhea	without antibiotic treatment with diarrhea	no antibiotic treatment and no diarrhea
491	17	11	5

Patient population: AB+DR+: Treated with antibiotics and had diarrhea; AB+DR-: Patients had antibiotic therapy without diarrhea; AB-DR+: Patients without antibiotic treatment with diarrhea; AB-DR-: Patients had no antibiotic treatment and were not suffering from diarrhea.

a *C. difficile* species-specific marker. Toxin profiling of isolates was fulfilled by RealStar® *C. difficile* PCR Kit 1.0 (Altona) as described below.

2.Cytotoxicity assay

Monolayer cultures of Hep-2 cells were prepared in 96-well plates (Jet-Biofil®) using RPMI (Gibco®) supplemented with 5% FBS (Gibco®), non-essential amino acids (Gibco®), and penicillin-streptomycin (Gibco®) (12). Three colonies from GDH *C. difficile* isolates were grown in 30mL of TSB media (Trypticase Soy Broth) under anaerobic conditions for 72h. Cell-free media were subsequently prepared by centrifuging cultures at 8000×g for 20 min at 4°C. The cultures were then passed through 0.22 µm membrane filters. Monolayer Hep-2 cell cultures were directly exposed to *C. difficile* supernatants for 30, 60, and 120 min, and cell viability was measured using the MTT test. Briefly, culture supernatants were removed, and wells were washed with fresh RPMI medium. Then, complete RPMI media (above) which contained MTT 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5mg/mL) was added to all wells and incubated for 4h. Plates were then centrifuged (8000×g) to remove media, and the purple formazan crystals were dissolved by adding 100 µL of DMSO (Sigma-Aldrich) to each well. The absorbance was measured at 570 nm using mQuant plate reader (Bio-Tek Instruments), and cell viability was calculated according to the following equation (13):

Cell viability (%)=((absorbance of treated well))/((absorbance of control well))×100

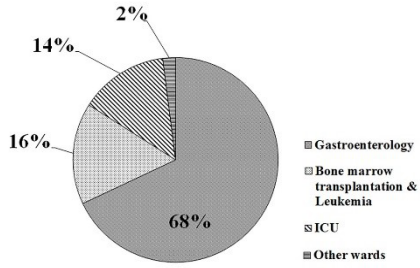
Real-time PCR assay for detection of *tcd*A and *tcd*B

We used RealStar®*C. difficile* PCR Kit 1.0 of the Altona company (Germany) for qualitative *C. difficile* toxin A and toxin B multiplex real-time PCR assay using the manufacturer protocols. The real-time amplification experiments were performed using LightCycler@ 96 (Roche) and Rotor-gene Q (Qiagen) machines simultaneously to ensure the accuracy of the results. Real-time PCR data were analyzed by LightCycler@ 96 software version 1.1.0.1320 or Rotor-gene Q 6000 software according to the machine used.

All stool samples were inoculated with the internal control template from RealStar® *C. difficile* PCR Kit to ensure the accuracy of DNA extraction and amplification, which was subsequently evaluated with the primer-probe set provided in the kit. Extracted DNA from stool samples was simultaneously tested as duplicate. Proper positive and negative controls were considered in all test runs.

RealStar® *C. difficile* PCR assay was performed with the following reaction mixture: RealStar® master mix A (5µL/reaction), master mix B (15µL/reaction), and eluate from nucleic acid extraction (10µL/reaction). Amplification was performed on both machines simultaneously following the kit protocol: 2 min at 95°C, followed by 45 cycles consisting of 15 sec at 95°C and 45 sec at 58°C for a total combined amplification and detection time of 90min.

Probes specific to *C. difficile* toxin genes, *tcd*A, and *tcd*B, and for internal control, were labeled Cy®5, FAM, and JOE fluorophores, respectively. The qualitative aspect of the real-time PCR defines a sample as positive if the Cy®5 (tcdA), FAM (tcdB),



The prevalence of C. difficile in clinical samples from antibiotic-associated diarrheal (AAD) patients hospitalized in various wards.

Figure.1: Prevalence of *C. difficile* infection.

Table 2: Comparison of toxigenic culture and cytotoxicity assay results with commercial *C. difficile* detection

A					
Assay results (RealStar® *C. difficile* PCR)	**Toxigenic culture + cytotoxicity assay +**	**Toxigenic culture + cytotoxicity assay –**	**Toxigenic culture - cytotoxicity assay –**	**Toxigenic culture + cytotoxicity assay –**	***Toxigenic culture - cytotoxicity assay -***
	GDH (gluD)+	**GDH (gluD)+**	**GDH (gluD)+**	**GDH (gluD) -**	**GDH (gluD) -**
tcdA+/tcdB+	34	0	9	0	0
tcdA+/tcdB-	0	0	0	0	0
tcdA-/tcdB+	6	0	0	0	0
tcdA-/tcdB-	0	13	0	4	424
Total (n=491)	40	13	9 (False Neg.)	4 (False Pos.)	424

and JOE (positive control) channels are positive. It is defined as negative if the Cy®5 and FAM channel are negative and the JOE channel is positive and undetermined and a retest is needed if all channels are negative.

RESULTS

Patient samples and population

Positive results were obtained from AB+DR+ population of patients (n=491) who had a history of antibiotic therapy and suffering from diarrhea; most were hospitalized in Gastroenterology, Bone Marrow Transplantation, and Leukemia wards (table 1, figure 1).

Cytotoxigenic culture assays

All stool specimens were cultured through the anaerobic culture method, and the isolates were confirmed as *C. difficile* by PCR-tracing of the GDH gene. We determined 53 *C. difficile* isolates and also four colonies as false positives. Cytotoxicity test confirmed 40 (8.1%) isolates as toxin-producing *C. difficile*. On the other hand, there were 13 non-toxigenic colonies (Table 2-A). The level of cytotoxin produced was qualitatively determined as a percentage of the number of dead or deformed cells in cell culture on Neobar lam. Considering the effect of the supernatant on the Hep-2 cells, they were classified into three categories of cells, which were fusiform in shape, normal, and alive. Also, cells were not spindle-shaped and had rounded shapes, but they were alive. These two forms were in the first group. The second group consisted of dead cells or cellular debris. Living cells were included in groups I and II, and the cytotoxin effect was compared with control samples

.Table 2: Comparison of toxigenic culture and cytotoxicity assay results with commercial *C. difficile* detection

A

Assay results (RealStar® *C. difficile* PCR)	Toxigenic culture + cytotoxicity assay +	Toxigenic culture + cytotoxicity assay –	Toxigenic culture - cytotoxicity assay –	Toxigenic culture + cytotoxicity assay –	*Toxigenic culture - cytotoxicity assay -*
	GDH (gluD)+	GDH (gluD)+	GDH (gluD)+	GDH (gluD) -	GDH (gluD) -
tcdA+/tcdB+	34	0	9	0	0
tcdA+/tcdB-	0	0	0	0	0
tcdA-/tcdB+	6	0	0	0	0
tcdA-/tcdB-	0	13	0	4	424
Total (n=491)	40	13	9 (False Neg.)	4 (False Pos.)	424

B

Results / Statistic	Toxigenic culture & Cytotoxicity assay		RealStar® C. difficile PCR assay	
	Values	95% CI	Values	95% CI
True positive	40		49	
True negative	424		442	
False positive	4		0	
False negative	10		0	
Sensitivity	80.00%	66.28 to 89.97%	100.00%	92.75 to 100%
Specificity	99.07%	97.62 to 99.74%	100.00%	99.17 to 100%
Positive Predictive Value%	90.91%	78.87 to 96.40%	100.00%	
Negative Predictive Value%	97.70%	96.00 to 98.68%	100.00%	
Accuracy	97.07%	97.13 to 98.39%	100.00%	99.25 to 100%

(A) Comparison of the RealStar® *C. difficile* PCR assay against toxigenic culture and cytotoxicity assay for detection of *Clostridium difficile*.
(B) Analytical sensitivity and specificity results of the positive toxin types detected; Toxigenic culture confirmed by Cytotoxicity assay and RealStar® *C. difficile* PCR assay

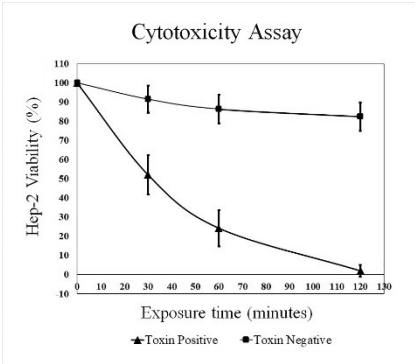
(14). The cytotoxicity test showed that toxigenic *C. difficile* culture supernatants killed the Hep-2 cells for up to 120 min while for those non-toxigenic isolates, a significantly lower decrease in cell culture viability was recorded (P<0.001) (figure 2).

Detection of the toxin genes by multiplex real-time PCR

A total of 49 (9.9%) toxin-producing *C. difficile* *tcd*A and *tcd*B toxin genes were characterized by RealStar® *C. difficile* PCR kit from 491 specimens. Among them, 40 were toxigenic culture positive, while the method did not detect nine toxin-positive *C. difficile* from fecal specimens. Among the toxin-producing *C. difficile* colonies, 43 were positive for both *tcd*A and *tcd*B (A+B+; 9.7%), and six were *tcd*B positive (A-B+; 1.4%) (table 2-A).

Analytical sensitivity and specificity

The analytical sensitivity and specificity of RealStar® *C. difficile* PCR kit and toxigenic culture procedure were compared along with positive predictive value (PPV), and negative predictive value (NPV), which is detailed in table 2-B. The results obtained from each run on both machines were analyzed through one-way analysis of variance (ANOVA), pairwise two-tailed correlation, and regression with SPSS software version 25.0 (IBM® SPSS® Statistics, USA) and showed no significant difference between results.



All *C. difficile* organisms isolated from stool samples of diarrhetic patients with a history of antibiotic treatment were assessed for cytotoxicity using Hep-2 cells as a target. Hep-2 cell cultures were exposed to acellular filtrates of bacterial broth culture, and then the viability of each culture was determined by MTT method. Cells exposed to supernatants of cytotoxic isolates showed a significant decrease in viability as compared with those exposed to non-toxigenic supernatants (P<0.001).

Figure.2: Cytotoxicity assessment of C. *difficile* isolates.

DISCUSSION

C. difficile infections are a pressing problem in developed countries, including the United States and Europe, as well as in developing countries. However, in developing countries, a lack of effective diagnostics and reporting procedures makes disease control very difficult(15-17). In addition, it is not routine to isolate the anaerobic spore-forming *C. difficile* in general clinical laboratories, and so this pathogen usually goes undetected (18).

Although antibiotic-associated diarrhea is a sign frequently encountered in clinical studies, it needs more practice and differential detection of *C. difficile* infection in Iranian hospitals (19-21).

In our survey, stool samples were collected from diarrheic inpatients with a history of antibiotic therapy from 2 to 8 weeks. The patients' population was mostly between critical wards of gastroenterology, intensive care unit, and bone marrow transplantation. We isolated 53 *C. difficile* colonies through an anaerobic culture of 491 stool samples and confirmed them by PCR-tracing the GDH gene (gluD), which were toxigenic or non-toxigenic types. This method resulted in the exclusion of four colonies as false positives, which indicates the low specificity of the culture method. Our MTT-based cytotoxicity assessment confirmed 40 isolates as toxin-producing *C. difficile*. Toxigenicity was investigated by exposing Hep-2 cells directly to the bacterial broth-culture filtrates. Although the phenotypic evaluation of toxin production is time-consuming and necessitates specialized laboratory equipment, the multiplex real-time PCR method can quantify the toxigenicity of the isolates and distinguish between low and high toxin-producing organisms, which may contribute to their pathogenicity (22).

Molecular methods, mainly nucleic acid amplification-based procedures, are gradually replacing most of the culture-based or enzymatic methods for diagnosing *C. difficile* infections. Molecular detection procedures speed-up of the CDI detection and serve to significantly improve sensitivity and specificity (23). In this study, we detected 49 (9.9%) toxigenic *C. difficile* by multiplex real-time PCR, which overlaps with the results obtained from anaerobic culture and cytotoxicity tests. It is noteworthy that nine (1.8%) toxigenic *C. difficile* were detected from patient stool samples by real-time PCR that had been missed by toxigenic culture, revealing the inefficiency of anaerobic culture as the gold standard for the detection of this globally important pathogen. Comparing toxigenic culture combining cytotoxicity assay against the multiplex real-time PCR showed that the RealStar® *C. difficile* qualitative PCR assay had the sensitivity and specificity of 100%, by considering toxin-producing *C. difficile* as the main parameter. Analytical sensitivity and specificity of the toxigenic culture method were 80% and 99.07%, respectively, which is by far lower than the molecular method used in our work.

C. difficile toxins A and B are considered to be of critical importance in the pathogenesis of the

organism, and toxin profiling for each patient may help for more effective treatment of each individual (24). Here we determined the toxigenicity profile of each detected *C. difficile* organism by multiplex real-time PCR, which showed that the predominant profile was *tcd*A+ *tcd*B+ (43; 9.7%), which is consistent with previous reports from all over the globe(25,26). Other profiles were one 6 *tcd*B+ (1.4%), showing less prevalent toxin-producing organisms.

Based on our findings, the prevalence of toxigenic *C. difficile* in patients with antibiotic-associated diarrhea was 9.9%. Previous reports from studies in the recent decade show that the prevalence of CDI rate in Iranian healthcare systems or hospitals ranges from 6% to 21%, which are obtained mostly through ELISA or PCR (27,28). To our knowledge, this is the first study using real-time PCR for tracing the pathogen in clinical specimens. Moreover, in the present work, we compared the commercial RealStar® *C. difficile* PCR kit with the most precise methods recommended in the guidelines (5,29).

In the past decade, the prevalence of CDI has increased all over the world. Most of the studies in Latin America encompassing AAD show a wide range of 8.3% to up to 38.5% of patients with CDI(30). Reports from South Africa show the range of 11.4% to 17.2% infections with toxigenic *C. difficile* associated with AAD. Other reports focused on diarrhea in HIV-infected individuals and reported a prevalence of 8.6% to 43% in hospitalized patients in African countries (31). Although there are reports from Korea (18.3%), Qatar (7.9%)(26), there are limited data on the prevalence of toxigenic *C. difficile* in Middle-Eastern countries, which might be attributed to the lack of equipped and specialized laboratory settings for detection of such organisms.

Detection of *C. difficile* toxin A and B genes forms the cornerstone of our method for effective molecular detection of *C. difficile* infections (32). As shown by our data, detection of the toxigenic *C. difficile* through conventional anaerobic culture and subsequent tests are time-consuming and impractical in most clinical laboratories (33). Molecular detection of the pathogenic types of the *C. difficile* by tracing the toxin genes directly from fecal specimens speeds up the diagnosis and, by far, is more accurate and practical in most of clinical laboratories, requiring a minimal knowledge and experience of molecular diagnosis and specialized equipment.

The current study was conducted in a large hospital setting, and we collected samples from AAD patients who were hospitalized in various wards. The CDI is mostly an infection that accompanies underlying complications, and prolonged diagnostic procedures affect the patients' health (34). The model of our survey confirms that real-time PCR yields accurate results for the diagnosis of C. difficile infections in a very short time, and the data are reliable for medical professionals to manage patients.

In conclusion, to improve *C. difficile* infection diagnosis, it is needed to clarify the accuracy and also the availability of procedures of interest. Two-stage algorithms have been suggested in all valid guidelines for assembling CDI diagnostic strategy. Anyhow, the conformity of laboratory results and the clinical picture should be approved.

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Contribution

SK, AMM, AV and MS are contributed to the designing and execution of the experiments and data analysis. SK and NK contributed to the preparation of the manuscript; MKF and SK contributed to cytotoxicity assessment and cell culture experiments.

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Ethics

This survey was approved by the Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1395.403) and Digestive Disease Research Institute, Tehran University of Medical Sciences.

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