ORIGINAL ARTICLE

Pathogenicity locus determinants and toxinotyping of *Clostridioides difficile* isolates recovered from Iranian patients

A. Aliramezani[1], M. Talebi[9], A. Baghani[1], M. Hajabdolbaghi[2], M. Salehi[2], A. Abdollahi[3], S. Afhami[4], M. Marjani[10], F. Golbabaei[11], M. A. Boroumand[5], A. Sarrafnejad[6], M. Yaseri[7], S. Ghourchian[1] and M. Douraghi[1,8]

1) *Division of Microbiology, Department of Pathobiology, School of Public Health,* 2) *Department of Infectious Diseases and Tropical Medicine, Faculty of Medicine,* 3) *Department of Pathology, Imam Hospital Complex,* 4) *Department of Infectious Diseases, Shariati Hospital,* 5) *Department of Pathology, Tehran Heart Center,* 6) *Department of Immunology, School of Public Health,* 7) *Department of Epidemiology and Biostatistics, School of Public Health,* 8) *Food Microbiology Research Center, Tehran University of Medical Sciences,* 9) *Department of Microbiology, School of Medicine, Iran University of Medical Sciences,* 10) *Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences and* 11) *Department of Occupational Health, School of Public Health, Medical Sciences, University of Tehran, Tehran, Iran*

Abstract

Little is known about the toxin profiles, toxinotypes and variations of toxin *Clostridioides difficile* C (*tcdC*) in Iranian *C. difficile* isolates. A total of 818 stool specimens were obtained from outpatients (n = 45) and hospitalized patients (n = 773) in Tehran, Iran, from 2011 to 2017. The 44 *C. difficile* isolates were subjected to PCR of toxin *C. difficile* A (*tcdA*), toxin *C. difficile* B (*tcdB*), *tcdA* 3′-end deletion, toxinotyping and sequencing of the *tcdC* gene. Thirty-eight isolates (86.36%) were identified as *tcdA* and *tcdB* positive, and the remaining six isolates (13.63%) were nontoxigenic. All *tcdA*- and *tcdB*-positive isolates yielded an amplicon of 2535 bp by PCR for the *tcdA* 3′ end. Fourteen (36.84%), seventeen (44.73%) and seven (18.43%) isolates belonged to wild-type, toxin *C. difficile* C subclone3 (*tcdC-sc3*) and *tcdC-A* genotype of *tcdC*, respectively. Thirty-one isolates (81.57%) belonged to toxinotype 0, and seven isolates (18.42%) were classified as toxinotype V. This study provides evidence for the circulation of historical and hypervirulent isolates in the healthcare and community settings. Furthermore, it was also demonstrated that the *tcdC-A* genotype and toxinotype V are not uncommon among Iranian *C. difficile* isolates.

Keywords: *Clostridioides difficile*, *tcdA*, *tcdB*, *tcdC*, toxinotyping
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Corresponding authors: M. Talebi, Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran; and M. Douraghi, Division of Microbiology, Department of Pathobiology, School of Public Health, PO Box 14155-6446, Tehran University of Medical Sciences, Tehran, Iran.
E-mails: Talebi_25@yahoo.com **(M. Talebi),** mdouraghi@tums.ac.ir **(M. Douraghi)**

Introduction

Historically known as a primary aetiologic agent of nosocomial antibiotic-associated diarrhoea, *Clostridioides difficile* has recently emerged in community settings [1–3]. *C. difficile* infections are toxin mediated and are manifested clinically as a spectrum of mild to life-threatening symptoms, from diarrhoea to pseudomembranous colitis [4]. An enterotoxin (toxin A, TcdA) and a cytotoxin (toxin B, TcdB) are the main virulence determinants of *C. difficile* [5]. The cytotoxic activity of TcdB can lead to diarrhoea, while progression of illness and initial damage of colon are attributed to the enteropathy effects of TcdA [6]. Although the majority of toxigenic strains harbour TcdA and TcdB (TcdA positive/TcdB positive), a proportion of strains carry only TcdB (TcdA negative/TcdB positive) [7].

The genes encoding TcdA and TcdB are located on the 19.6 kb pathogenicity locus (PaLoc), which also contains three open reading frames including toxin *C. difficile* E (*tcdE*), toxin *C. difficile* R (*tcdR*) and *tcdC*. TcdC plays an important role as negative regulator of TcdA and TcdB production [8]. Various

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alterations have been found in the PaLoc genes of *C. difficile* strains throughout the world, and these variations have remarkable consequences on the structure and function of TcdA and TcdC proteins. A notable alteration is the deletion of 1.8 kb within the 3′ end of *tcdA* gene which gives rise to the formation of TcdA-negative/TcdB-positive *C. difficile* strains [9]. While such strains are potentially toxigenic, they could not be detected by cytotoxicity assays because truncated TcdA lacks the ligand-binding domain [7]. Changes in the C terminus of TcdA (A3 fragment) and the N terminus of TcdB (B1 fragment) toxins lead to the definition of 34 variants toxinotypes (I to XXXIV). The most important toxinotypes that were isolated from humans are toxinotype 0, III, IV, V and VIII. The nucleotide polymorphisms in *tcdC* gene including mutations and/or deletions in coding regions may lead to premature stop codons and consequently truncation of the functional TcdC protein. The mutated TcdC might be associated with increased production of TcdA and TcdB, and accordingly the virulence of *C. difficile* [10]. Little is known about the toxin profiles, toxinotyping, and variations of *tcdC* in of Iranian *C. difficile* strains. Therefore, we analysed the toxin profiles and variations in *tcdA* and *tcdC* genes of *C. difficile* strains recovered from patients with diarrhoea.

Materials and methods

Setting and isolates

This study was conducted at the anaerobic bacteriology laboratory affiliated with the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. A total of 818 stool specimens were obtained from outpatients (n = 45) and hospitalized patients (n = 773). These patients were suspected of having *C. difficile*–associated diarrhoea and were referred to the anaerobic bacteriology laboratory from 17 referral tertiary hospitals or clinics located in different geographical areas of Tehran, Iran, from 2011 to 2017 (Table 1). After alcohol shock, stools were cultivated on cycloserine cefoxitin fructose agar and were incubated anaerobically at 37°C for 48 hours. The suspected colonies were identified as *C. difficile* by colony morphology, specific horse odor, Gram staining and proline–aminopeptidase test [11].

PCR assays

Genomic DNA extraction of *C. difficile* isolates was done using Chelex 100 (Bio-Rad, Hercules, CA, USA) [12]. For molecular identification of *C. difficile* isolates, we used gene-specific primers targeting *C. difficile* housekeeping genes including triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gluD*), *C. difficile* upstream 2 (*cdu2*) and *C. difficile* downstream 3 (*cdd3*) genes [13–15]. *C. difficile* isolates were also screened for toxin A (*tcdA*) and toxin B (*tcdB*) genes [15,16]. To confirm complete absence of PaLoc, all *tcdA*- and *tcdB*-negative strains were tested with PCR using Lok1-Lok3 primers [17]. In addition, *tcdA* 3′ end (*tcdA*3′) deletion analysis was performed using NK9 and NKV011 primers [18]. The entire *tcdC* gene of isolates was amplified using C1 and C2 primers [16], and subsequently the PCR products were subjected to sequencing.

Toxinotyping

All *tcdA*- and *tcdB*-positive isolates were subjected to toxinotyping using A3 and B1 primers that were previously described [19].

Toxigenic culture

The toxigenic culture of *C. difficile* isolates was performed as follows: three to five colonies of a pure culture of bacteria were subcultured on brain–heart infusion broth and incubated anaerobically for 3 to 5 days at 37°C. After centrifugation and filtration, brain–heart infusion supernatant containing toxin was added to a 96-well microplate containing 10^4 Vero cell line. After examination of the cell line at 24 and 48 hours under 5% CO_2 at 37°C incubation conditions, cytopathic effects were recorded if 50% or more of the Vero cells were rounded [20].

Nucleotide sequence accession number

The nucleotide sequences of *tcdC* gene variants including wild type, truncated variant *tcdC-A* allele and *tcdC-sc3* allele were deposited in GenBank under the accession numbers, indicated in Table 2.

Results

Of 818 stool samples from outpatients and hospitalized patients, 44 isolates (5.37%) were identified as *C. difficile* based on detection of *tpi, gluD, cdu-2* or *cdd-3* (Table 1). Mean and standard deviation of patient age was 53.89 ± 22.44 years. Of 44 isolates, 38 (86.36%) were *tcdA* and *tcdB* positive and the remaining 6 (13.63%) isolates were *tcdA* and *tcdB* negative and nontoxigenic. All *tcdA*- and *tcdB*-negative isolates were positive in PCR reaction using Lok1-Lok3 primers and had 769 bp amplicon (Table 1).

Of the 38 *tcdA*- and *tcdB*-positive isolates, all isolates yielded an amplicon of 2535 bp by PCR amplification for the *tcdA* 3′ end, thus confirming no deletion at this region. Using NK9 and NKV011 primers, six isolates that were *tcdA* and *tcdB* negative also were negative in *tcdA* 3′-end analysis. Of 38 toxigenic isolates, 31 isolates (81.57%) belonged to toxinotype 0, and 7 (18.42%) were classified as toxinotype V (Table 1).

TABLE 1. Genetic profiles and molecular characteristic of *Clostridioides difficile* isolates

No.	Strain	Year of isolation	Source[a]	*cdu2/tpi/cdd3/gluD*	*tcdA/tcdB*	Lok1/Lok3	*tcdA* 3′ size (bp)	*tcdC*	*tcdC* deletion (bp)	*tcdC* stop codon at 184 bp	CPE	Toxinotype
1	PC002	2014	H	+/+/+/+	+/+	−	2535	+	39	+	+	V
2	PC004	2014	H	+/+/+/+	+/+	−	3100[b]	+	−	−	+	0
3	PC006	2014	H	+/+/+/+	+/+	−	2535	+	−	−	+	V
4	PC008	2014	H	−/+/+/+	+/+	−	3100[b]	+	39	+	+	0
5	PC009	2014	H	+/+/+/+	+/+	−	3100[b]	+	39	+	+	0
6	PC010	2014	H	+/+/+/+	+/+	−	2535	+	−	−	+	V
7	PC020	2014	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
8	PC021	2014	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
9	PC024	2014	H	+/+/+/+	+/+	−	3100[b]	+	−	−	+	0
10	PC028	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
11	PC035	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
12	PC036	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
13	PC048	2015	H	+/+/+/+	−/−	+	—	−	−	−	−	-
14	PC049	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
15	PC054	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
16	PC056	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
17	PC062	2015	H	+/+/+/+	+/+	−	2535	+	39	+	+	V
18	PC063	2015	O	+/+/+/+	+/+	−	2535	+	−	−	+	0
19	PC066	2015	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
20	PC069	2016	O	−/+/+/+	+/+	−	2535	+	39	+	+	V
21	PC071	2011	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
22	PC073	2012	H	+/+/+/+	−/−	+	—	−	−	−	−	-
23	PC074	2011	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
24	PC075	2011	H	+/+/+/+	−/−	+	—	−	−	−	−	-
25	PC080	2016	H	+/+/+/+	−/−	+	—	−	−	−	−	-
26	PC087	2016	H	−/+/+/+	+/+	−	2535	+	−	−	+	0
27	PC089	2016	H	−/+/+/+	+/+	−	2535	+	−	−	+	0
28	PC091b	2016	H	+/+/+/+	−/−	+	—	−	−	−	−	-
29	PC092b	2016	H	−/+/+/+	+/+	−	2535	+	−	−	+	0
30	PC096	2016	H	−/+/+/+	+/+	−	2535	+	39	+	+	V
31	PC098	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
32	PC101	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
33	PC102	2016	H	+/+/+/+	−/−	+	—	−	−	−	−	-
34	PC103	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
35	PC106	2016	H	+/+/+/+	+/+	−	2535	+	39	+	+	V
36	PC107	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
37	PC111	2016	H	+/+/+/−	+/+	−	2535	+	−	−	+	0
38	PC112	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
39	PC113	2016	H	+/+/+/−	+/+	−	2535	+	−	−	+	0
40	PC114	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
41	PC115	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
42	PC116	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
43	PC117	2016	H	+/+/+/+	+/+	−	2535	+	−	−	+	0
44	PC118	2017	H	+/+/+/+	+/+	−	2535	+	−	−	+	0

[a]Hospitalized patients (H) or outpatients (O).
[b]Amplicon was obtained using A3C and A4N primers [19].

Among the toxigenic isolates, 14 *C. difficile* isolates (36.84%) had no deletion in *tcdC* sequences and were assigned to the wild-type *tcdC* genotype. Seventeen isolates (44.73%) contained a G → T transition at nucleotide 148 and belonged to *tcdC-sc3* genotype. Seven isolates (18.43%) had deletion of 39 bp and also a C → T transition at nucleotide 184, and represented the *tcdC-A* genotype. The latter transition is proposed to result in truncation of the TcdC protein (Table 2).

Discussion

We found that 5% of patients harboured *C. difficile* as either toxigenic or nontoxigenic isolates. This proportion is concordant with the previous report from Tehran, Iran, using enzyme-linked immunosorbent assay [21]. Using PCR for detection of *tcdA* and *tcdB,* we demonstrated that the majority of *C. difficile* isolates harboured *tcdA* and *tcdB* genes (Table 1). On the other hand, the isolates with no amplicon for *tcdA*/*tcdB* were confirmed to be nontoxigenic by a positive assay yielding an amplicon of 769 bp using Lok1 and Lok3 (Table 1) [17]. The frequency of toxigenic isolates tested in the current study (86.36%) was slightly higher than another study (84.2%) reported from Tehran, Iran [22]. In neighbouring countries such as Kuwait, the rate of toxigenic *C. difficile* was reported to be 0.54% to 64.6% [23,24]. This difference might be partly related to the sample size, the target population and mainly to the primer set used in the current study. We used the primers targeting the 5′ end of the *tcdA* gene [15] and amplifying the conserved region and nonrepeating fragment of *tcdA*. Using this set of primers, all but the nontoxigenic isolates yielded amplicons, and as expected, the negative result for PCR was unlikely unless the isolates had a large deletion in *tcdA* [15]. Therefore, the isolates harbouring the *tcdA* gene was subsequently assayed for *tcdA* deletion in the 3′ end. Analysis of the 3′ end of *tcdA* revealed that all the isolates except four (PC004, PC008,

TABLE 2. ***tcdC*** **genotypes of** ***Clostridioides difficile*** **isolates**

No.	Strain	Mutation (nucleic acid residues)	*tcdC* genotype	GenBank accession no.
1	PC002	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675257
2	PC004	G → T (148)	*tcdC-sc3*	MG675248
3	PC006	G → T (148)	*tcdC-sc3*	MG675249
4	PC008	—	Wild type	MG675253
5	PC009	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675258
6	PC010	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675259
7	PC020	G → T (148)	*tcdC-sc3*	MG569922
8	PC021	G → T (148)	*tcdC-sc3*	MG675250
9	PC024	—	Wild type[a]	MG596349
10	PC028	—	Wild type	MG596350
11	PC035	G → T (148)	*tcdC-sc3*	MG655373
12	PC036	G → T (148)	*tcdC-sc3*	MG655374
13	PC049	G → T (148)	*tcdC-sc3*	MG655375
14	PC054	G → T (148)	*tcdC-sc3*	MG675251
15	PC056	G → T (148)	*tcdC-sc3*	MG655376
16	PC062	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG581978
17	PC063	G → T (148)	*tcdC-sc3*	MG655377
18	PC066	G → T (148)	*tcdC-sc3*	MG675238
19	PC069	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675239
20	PC071	G → T (148)	*tcdC-sc3*	MG675240
21	PC074	—	Wild type	MG675241
22	PC087	G → T (148)	*tcdC-sc3*	MG675242
23	PC089	—	Wild type	MG675243
24	PC092b	—	Wild type	MG675244
25	PC096	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675245
26	PC098	G → T (148)	*tcdC-sc3*	MG675246
27	PC101	G → T (148)	*tcdC-sc3*	MG675247
28	PC103	—	Wild type	MG675255
29	PC106	G → T (53)/A → T (117)/C → T (120)/C → T (183)/C → T (184) (stop codon)/A → G (330)/G → T (430)/ A → C (516)/T → A (558) (stop codon)/T → A (585)/T → C (660)/39bp deletion (341–379)	*tcdC-A*	MG675260
30	PC107	—	Wild type	MG675256
31	PC111	—	Wild type	MG788278
32	PC112	—	Wild type	MG788279
33	PC113	—	Wild type	MG788280
34	PC114	G → T (148)	*tcdC-sc3*	MG788284
35	PC115	G → T (148)	*tcdC-sc3*	MG788285
36	PC116	—	Wild type	MG788281
37	PC117	—	Wild type	MG788282
38	PC118	—	Wild type	MG788283

CPE, cytopathic effects.
[a]Published sequence of *Clostridioides difficile* strain VPI10463 was used as reference strain for comparison of all sequences [34].

PC009, PC024) produced an amplicon of 2535 bp using primers NK9-NKV011 (Table 1) [18]. While no amplicon was observed for the latter four isolates in PCR of the 3′ end, the amplicons of 3100 bp were noted using the primers directed at the A3 fragment used for toxinotyping [19]. Altogether, the isolates with a 2535 bp amplicon or 3100 bp were considered ToxA+/ ToxB+ (Table 1).

Toxinotyping of *tcdA*- and *tcdB*-positive isolates showed that these isolates belonged to toxinotype 0 or V. The most predominant toxinotype in our study was toxinotype 0, and this toxinotype showed no changes in *tcdA* and *tcdB* gene sequences (Table 1) [19]. Previous studies indicated that the *C. difficile* isolates with the entire repeating region of *tcdA* had toxinotype 0 and V [25,26]. Jalali et al. [27] also found that toxinotype 0 was the prevalent toxinotype in an Iranian hospital. The most frequent toxinotypes in Asia are toxinotype 0 and VIII [24,28]. Two studies reported that 71.4% and 7.69% of *C. difficile* toxinotypes in different hospitals in Kuwait and Lebanon belonged to toxinotype 0, respectively [24,29]. These data show the minor changes in PaLoc either in Iran or Asia.

With respect to clinical manifestations, either the nontoxigenic or toxigenic isolates were recovered from symptomatic patients who had diarrhoea. One possible explanation for recovery of the nontoxigenic *C. difficile* isolates might be the presence of such isolates as a member of intestinal microbiota [30]. In other words, the clinical manifestations may not be associated with the colonization of intestine by *C. difficile* or its carriage by patients, and only the intake of antibiotics may contribute to the development of antibiotic-associated diarrhoea [31]. For instance, one of the nontoxigenic isolates was recovered from a 54-year-old woman with HIV and toxoplasmosis. This patient was hospitalized for a long period, was subjected to the antimicrobial therapies and finally died. The patient had several predisposing factors, but it is difficult to conclude whether the nontoxigenic isolate was significantly implicated in diarrhoea. Another explanation is that apart from toxins, other virulence factors of *C. difficile,* particularly the colonization factors, may

induce a pathologic response in vulnerable patients [32] and consequently give rise to disease. It has been reported that up to 50% of *C. difficile* isolated from healthy volunteers and asymptomatic hospitalized patients were nontoxigenic strains. Although there are several case reports describing the possible role of nontoxigenic isolates as risk or protective factor [33], further studies are needed to assess the function of nontoxigenic isolates in inducing *C. difficile*–associated clinical outcomes.

We identified three types of *tcdC* genes in our isolates using sequencing: wild type, *tcdC-A* and *tcdC-sc3* genotypes. The *tcdC-A* genotype is characterized by the existence of nonsense mutation at nucleotide 184 and 39 bp deletion at nucleotides 341 to 379 [34]. Toxinotyping revealed that all the isolates that had 39 bp deletion in *tcdC* gene belonged to toxinotype V, except two isolates. The isolates with no changes in *tcdC* gene were classified as toxinotype 0 except two isolates that belonged to toxinotype V (Tables 1 and 2). Isolates with TcdC truncation and toxinotype V may cause severe infections in humans and animals and may be identified as hypervirulent strains [35]. Hypervirulent *C. difficile* strains also express binary toxins (*cdtA* and *cdtB*) that may increase the severity of disease [35]. Little is known about the heterogenicity of *C. difficile* toxin genes in Iranian isolates, especially in Tehran. Jalali et al. [27] found that 0, V and XXIV toxinotypes were predominant in Isfahan. In our study, the six isolates with *tcdC-A* genotype were also positive for binary toxin (*cdtA*, *cdtB*) except one isolate using gene-specific PCR (data not shown). These six *cdtA*- and *cdtB*-positive isolates also belonged to toxinotype V. Six isolates of *tcdC-A* genotype were obtained from hospitalized patients and one from an outpatient (Tables 1 and 2). Jalali et al. [27] also reported that the isolates that possess 39 bp deletion in *tcdC* gene belonged to toxinotype V isolated from hospitalized patients. Persson et al. [15] and Spigaglia et al. [16] reported that all the isolates that have a 39 bp deletion in *tcdC* gene may belong to toxinotypes V, VI and VII. Among the *tcdC* genotypes, the *tcdC-sc3* genotype has the highest frequency, as reported in other studies (Table 2) [34,]. This study provides molecular evidence that the isolates with either toxigenic or nontoxigenic profiles are circulating in the healthcare and community settings. Furthermore, it was also demonstrated that the *tcdC-A* genotype and toxinotype V is not uncommon among Iranian *C. difficile* isolates. This finding sheds light on the possibility of the contribution of hypervirulent isolates in *C. difficile* infections in addition to historical isolates of *C. difficile*.

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Conflict of interest

None declared.

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