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

A. Aliramezani¹, M. Talebi⁵, A. Baghani¹, M. Hajabdolbaghi³, M. Salehi⁵, A. Abdollahi¹, S. Afhami⁴, M. Marjani¹⁰, F. Golbabaei¹¹, M. A. Boroumand¹, A. Sarrafnejad⁶, M. Yaseri⁷, S. Ghourchian¹ and M. Douraghi¹,⁸

1) Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, 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, toxinoetypes 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, toxinootyping 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 nontoxicigenic. 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, 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 tcdCA genotype and toxinotype V are not uncommon among Iranian *C. difficile* isolates.

© 2018 Published by Elsevier Ltd.

**Keywords:** *Clostridioides difficile*, tcdA, tcdB, tcdC, toxinootyping

**Original Submission:** 13 May 2018; **Revised Submission:** 25 June 2018; **Accepted:** 5 July 2018

**Article published online:** 2 August 2018

**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
alterations have been found in the PaLoc genes of C. difficile strains throughout the world, and these variations have remarkable implications 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, toxino typing, 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 (gdUD), C. difficile upstream 2 (cdu2) and C. difficile downstream 3 (cd3) 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 (tcdA3’) 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.

Toxino typing
All tcdA- and tcdB-positive isolates were subjected to toxino typing 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% CO2 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, gdUD, cd2-2 or cd3 (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).
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 184, and represented the *tcdC* genotype. Seven isolates (18.43%) had deletion of 39 bp and also a C → T transition at nucleotide 184, and represented the *tcdC*-sc3 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, PC010, PC013) harboured a single nucleotide mutation (Table 2).
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 Tox A+/Tox B+ (Table 1).

Toxinotyping of tcdA- and tcdB-positive isolates showed that these isolates belonged to toxotype 0 or V. The most predominant toxotype in our study was toxotype 0, and this toxotype 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 toxotype 0 and V [25,26]. Jalali et al. [27] also found that toxotype 0 was the prevalent toxotype in an Iranian hospital. The most frequent toxotypes in Asia are toxotypes 0 and VIII [24,28]. Two studies reported that 71.4% and 7.69% of C. difficile toxotypes in different hospitals in Kuwait and Lebanon belonged to toxotype 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 significantly implicated in diarrhoea.

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

| 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 (640)/39bp deletion (341–379) | tcdCA | MG675257 |
| 2   | PC004  | G → T (148) | tcdScJ | MG675248 |
| 3   | PC006  | G → T (148) | tcdScJ | 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 (640)/39bp deletion (341–379) | tcdCA | 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 (640)/39bp deletion (341–379) | tcdCA | MG675259 |
| 7   | PC020  | G → T (148) | tcdScJ | MG69992 |
| 8   | PC021  | G → T (148) | tcdScJ | MG675250 |
| 9   | PC024  | — | Wild type | MG686349 |
| 10  | PC028  | — | Wild type | MG686350 |
| 11  | PC035  | G → T (148) | tcdScJ | MG655373 |
| 12  | PC036  | G → T (148) | tcdScJ | MG655374 |
| 13  | PC049  | G → T (148) | tcdScJ | MG655375 |
| 14  | PC054  | G → T (148) | tcdScJ | MG675251 |
| 15  | PC056  | G → T (148) | tcdScJ | 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 (640)/39bp deletion (341–379) | tcdCA | MG51978 |
| 17  | PC063  | G → T (148) | tcdScJ | MG655377 |
| 18  | PC066  | G → T (148) | tcdScJ | 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 (640)/39bp deletion (341–379) | tcdCA | MG675239 |
| 20  | PC071  | G → T (148) | tcdScJ | MG675240 |
| 21  | PC074  | G → T (148) | Wild type | MG675241 |
| 22  | PC087  | G → T (148) | tcdScJ | 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 (640)/39bp deletion (341–379) | tcdCA | MG675245 |
| 26  | PC098  | G → T (148) | tcdScJ | MG675246 |
| 27  | PC101  | G → T (148) | tcdScJ | 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 (640)/39bp deletion (341–379) | tcdCA | 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) | tcdScJ | MG788284 |
| 35  | PC115  | G → T (148) | tcdScJ | MG788285 |
| 36  | PC116  | — | Wild type | MG788281 |
| 37  | PC117  | — | Wild type | MG788282 |
| 38  | PC118  | — | Wild type | MG788283 |

© 2018 Published by Elsevier Ltd. NMNI, 25, 52–57
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 toxinoype V, except two isolates. The isolates with no changes in tcdC gene were classified as toxinoype 0 except two isolates that belonged to toxinoype V (Tables 1 and 2). Isolates with TcdC truncation and toxinoype 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 toxinoypes were predominant in Isfahan. In our study, the six isolates with tcdC-A genotypes were also positive for binary toxin (cdtA, cdtB) except one isolate using genome-specific PCR (data not shown). These six cdtA- and cdtB-positive isolates also belonged to toxinoype V. Six isolates of tcdC-A genotypes 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 toxinoype 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 toxinoypes V, VI and VII. Among the tcdC-A 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 toxinoype 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.

**Acknowledgement**

Supported in part by grant 31372 from Tehran University of Medical Sciences and Health Services.

**Conflict of interest**

None declared.

**References**

1. Johnson S. Editorial commentary: changing epidemiology of Clostridium difficile and emergence of new virulent strains. Clin Infect Dis 2014;58:1731–3.
2. Khan FY, Elzouki AN. Clostridium difficile infection: a review of the literature. Asian Pac J Trop Med 2014;7:56–13.
3. Ong GK, Reidy TJ, Huk MD, Lane FR. Clostridium difficile colitis: a clinical review. Am J Surg 2017;213:565–71.
4. Bartlett JG. Clostridium difficile infection. Infect Dis Clin North Am 2017;31:489–95.
5. Lewis BB, Carter RA, Ling L, Leiner I, Taur Y, Kamboj M, et al. Pathogenicity locus, core genome, and accessory gene contributions to Clostridium difficile virulence. mBio 2017;8. e00885–17.
6. Voth DE, Ballard JD. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev 2005;18:247–63.
7. Drudy D, Fanning S, Kyne L. Toxin A—negative, toxin B—positive Clostridium difficile. Int J Infect Dis 2007:11:5–10.
8. King AM, Mackin KE, Lyras D. Emergence of toxin A—negative, toxin B—positive Clostridium difficile strains: epidemiological and clinical considerations. Future Microbiol 2015;10:1–4.
9. Jank T, Beliy Y, Aktories K. Bacterial glycosyltransferase toxins. Cell Microbiol 2015;17:1752–65.
10. Persson S, Jensen JN, Olsen KE. Multiplex PCR method for detection of Clostridium difficile tcdA, tcdB, cdtA, and cdtB and internal in-frame deletion of tcdC. J Clin Microbiol 2011;49:4299–300.
11. Fedorko DP, Williams EC. Use of cycloserine-cefoxitin-fructose agar and l-proline-aminopeptidase (PRO Discs) in the rapid identification of Clostridium difficile. J Clin Microbiol 1997;35:1258–9.
12. Arroyo LG, Kruth SA, Willey BM, Stamperl HR, Low DE, Weese JS. PCR ribotyping of Clostridium difficile isolates originating from human and animal sources. J Med Microbiol 2005;54:163–6.
13. Lemes D, Dhalioum A, Testelin S, Matzrat MA, Mailard K, Lemeland JF, et al. Multiplex PCR targeting tpi (ribose phosphate isomerase), tcdA (toxin A), and cdtB (toxin B) genes for toxigenic culture of Clostridium difficile. J Clin Microbiol 2004;42:5710–4.
14. Zheng L, Keller S, Lyerly D, Carman R, Genheimer C, Kolesar J, et al. Multicenter evaluation of a new screening test that detects Clostridium difficile in fecal specimens. J Clin Microbiol 2004;42:3837–40.
15. Persson S, Torpsh M, Olsen K. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect 2008;14:1057–64.
16. Spigaglia P, Mastrandonto P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among Clostridium difficile clinical isolates. J Clin Microbiol 2002;40:3470–5.
17. Braun V, Hundsberger T, Leukel P, Sauerborn M, von Eichel-Streiber C. Definition of the single integration site of the pathogenicity locus in Clostridium difficile. Gene 1996;181:29–38.
18. Kato H, Kato N, Katow S, Maegawa T, Nakamura S, Lyerly DM. Deletions in the repeating sequences of the toxin A gene of toxin A—negative, toxin B—positive Clostridium difficile strains. FEMS Microbiol Lett 1999;175:197–203.
19. Rupnik M, Vesnaver V, Janc M, van Eichel-Streiber C, Delmée M. A novel toxinotyping scheme and correlation of toxinootypes with
serogroups of Clostridium difficile isolates. J Clin Microbiol 1998;36:2240–7.

[20] Barbut F, Kajzer C, Planas N, Petit JC. Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of Clostridium difficile–associated diarrhea. J Clin Microbiol 1993;31:963–7.

[21] Nazemalhosseini-Mojarad E, Azimirad M, Razgahi M, Torabi P, Moosavi A, Alebouyeh M, et al. Frequency of Clostridium difficile among patients with gastrointestinal complaints. Gastroenterol Hepatol Bed Bench 2011;4:210.

[22] Shayganmehr FS, Alebouyeh M, Azimirad M, Aslani MM, Zali MR. Association of tcdA+/tcdB+ Clostridium difficile genotype with emergence of multidrug-resistant strains conferring metronidazole resistant phenotype. Iran Biomed 2015;19:143.

[23] Jamal W, Rotimi V, Grubesic A, Rupnik M, Brazier J, Durden B. Correlation of multidrug resistance, toxinotypes and PCR ribotypes in Clostridium difficile isolates from Kuwait. J Chemother 2009;21:521–6.

[24] Kim SJ, Kim H, Seo Y, Yong D, Jeong SH, Chong Y, et al. Molecular characterization of toxin A–negative, toxin B–positive variant strains of Clostridium difficile isolated in Korea. Diagn Microbiol Infect Dis 2010;67:198–201.

[25] Rupnik M, Kato N, Grabnar M, Kato H. New types of toxin A–negative, toxin B–positive strains among Clostridium difficile isolates from Asia. J Clin Microbiol 2003;41:1118–25.

[26] Jalali M, Khorvash F, Warriner K, Weese JS. Clostridium difficile infection in an Iranian hospital. BMC Res Notes 2012;5:159.

[27] Barbut F, Kajzer C, Planas N, Petit JC. Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of Clostridium difficile–associated diarrhea. J Clin Microbiol 1993;31:963–7.

[28] Rupnik M, Janezic S. An update on Clostridium difficile toxotyping. J Clin Microbiol 2016;54:13–8.

[29] Moukhaiber R, Araj GF, Kissayan KAB, Cheaito KA, Matar GM. Prevalence of Clostridium difficile toxinotypes in infected patients at a tertiary care center in Lebanon. J Infect Dev Ctries 2015;9:732–5.

[30] Stojanovic P, Kocic B, Stojanovic M, Miljkovic-Selimovic B, Tasic S, Miladinovic-Tasic N, et al. Clinical importance and representation of toxigenic and non-toxigenic Clostridium difficile cultivated from stool samples of hospitalized patients. Braz J Microbiol 2012;43:215–23.

[31] Thomas C, Stevenson M, Riley TV. Antibiotics and hospital-acquired Clostridium difficile–associated diarrhoea: a systematic review. J Antimicrob Chemother 2003;51:1339–50.

[32] Vedantam G, Clark A, Chu M, McQuade R, Mallozzi M, Viswanathan V. Clostridium difficile infection: toxins and non-toxin virulence factors, and their contributions to disease establishment and host response. Gut Microbe 2012;7:10.

[33] Natarajan M, Walk ST, Young VB, Aronoff DM. A clinical and epidemiological review of non-toxigenic Clostridium difficile. Anaerobe 2013;22:1–5.

[34] Curry SR, Marsh JW, Muto CA, O’Leary MM, Pascelle AW, Harrison LH. tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of Clostridium difficile. J Clin Microbiol 2007;45:215–21.

[35] Goldenberg SD, French GL. Lack of association of tcdC type and binary toxin status with disease severity and outcome in toxigenic Clostridium difficile. J Infect 2011;62:353–62.