Molecular Characterization of Pathogenicity Locus (PaLoc) and tcdC Genetic Diversity Among tcdA^+B^+ Clostridioides Difficile Clinical Isolates in Tehran, Iran

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Research

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Abstract

**Background:** *Clostridioides difficile* is the main cause of healthcare-associated diarrhea worldwide. It is proposed that certain *C. difficile* toxinotypes with distinct pathogenicity locus (PaLoc) variants are associated with disease severity and outcomes. Additionally, few studies have described the common *C. difficile* toxinotypes, and also little is known about the tcdC variants in Iranian isolates. We characterized the toxinotypes and the tcdC genotypes from a collection of Iranian clinical *C. difficile* tcdA+B+ isolates with known ribotypes (RTs).

**Methods:** Fifty *C. difficile* isolates with known RTs and carrying the tcdA and tcdB toxin genes were analyzed. Toxinotyping was carried out based on a PCR-RFLP analysis of a 19.6 kb region encompassing the PaLoc. Genetic diversity of the tcdC gene was determined by the sequencing of the gene.

**Results:** Of the 50 *C. difficile* isolates investigated, five distinct toxinotypes were recognized. Toxinotypes 0 (33/50, 66%) and V (11/50, 22%) were the most frequently found. *C. difficile* isolates of the toxinotype 0 mostly belonged to RT 001 (12/33, 36.4%), whereas toxinotype V consisted of RT 126 (9/11, 81.8%). The tcdC sequencing showed six variants (35/50, 70%); tcdC-sc3 (24%), tcdC-A (22%), tcdC-sc9 (18%), tcdC-B (2%), tcdC-sc14 (2%), and tcdC-sc15 (2%). The remaining isolates were wild-types (15/50, 30%) in the tcdC gene.

**Conclusions:** The present study demonstrates that the majority of clinical tcdA+B+ isolates of *C. difficile* frequently harbor tcdC genetic variants. We also found that the RT 001/ toxinotype 0 and the RT 126/ toxinotype V are the most common types among Iranian isolates. Further studies are needed to investigate the putative association of various tcdC genotypes with CDI severity and its recurrence.

Background

*Clostridioides* (formerly *Clostridium*) *difficile* is the leading cause of hospital-acquired diarrhea (HAD) with notable morbidity and mortality worldwide [1, 2]. This bacterial pathogen causes toxin-mediated diseases ranging from self-limited diarrhea to severe pseudomembranous colitis (PMC) [3]. Over the last two decades, the incidence of *C. difficile* infection (CDI) has rapidly increased due to the emergence of hypervirulent epidemic types [4]. Nowadays, the increasing number of initial episodes of CDI and recurrent CDI (rCDI) can result in a higher economic healthcare burden [5].

Alteration of the gut microbiota, due to antibiotic therapy, chemotherapy, advanced age and prolonged hospitalization, is the main risk factor for CDI development [6–8]. The pathogenicity of CDI is linked to the production of toxin A (TcdA) and toxin B (TcdB); the key virulence factors [9]. The toxin encoding genes are located on a chromosomal element called the pathogenicity locus (PaLoc). Furthermore, PaLoc encodes three accessory genes; positive and negative regulators (tcdR and tcdC, respectively) and a holin-like gene (tcdE) that has been shown to be involved in toxin release from *C. difficile* cells [10, 11]. However, the exact role of the TcdE protein in the release of *C. difficile* toxins has been controversial [12]. Moreover, it was suggested that TcdE might be associated with early toxin release, since the TcdE-
dependent toxin secretion mechanism is regulated by different growth conditions [13, 14]. Also, it has been suggested that mutations of the \textit{tcdC} gene, which result in a truncation of the TcdC protein, leads to elevated toxin production in hypervirulent \textit{C. difficile} isolates such as BI/NAP1/027, although its role in toxin production is still a matter of controversy [15–17].

About 23\% of \textit{C. difficile} strains produce a third, binary toxin (CDT) which encodes in a separate region of the chromosome (CdtLoc) and consists of \textit{cdtA} and \textit{cdtB} genes for both subunits of CDT and a regulatory gene (\textit{cdtR}) [18]. The major virulence attributes of \textit{C. difficile} are TcdA and TcdB yet recent studies proposed that CDT could increase the severity of CDI in some of the most hypervirulent strains [19].

The genome of \textit{C. difficile} shows substantial interspecies heterogeneity particularly in the PaLoc of variant toxinotypes [20, 21]. Molecular characterization of the \textit{C. difficile} strains via toxinotyping and ribotyping has been widely used for phylogenetic and epidemiologic studies of CDI [22–24]. Moreover, the increased availability of molecular typing of isolates in different geographic regions could improve our understanding of CDI epidemiology, and also for the development of molecular diagnostic tests and vaccines.

Little is known about the molecular typing of \textit{C. difficile} isolates in Iranian diarrheal patients. Additionally, the status of the \textit{tcdA}+\textit{B}+ \textit{C. difficile} isolates is not well analyzed in terms of toxinotyping and ribotyping. Thus, the main goal of the present study was to investigate the toxinotypes and genetic diversity of \textit{tcdC} gene in a collection of \textit{C. difficile} \textit{tcdA}+\textit{B}+ isolates with known ribotype derived from hospitalized patients in Tehran healthcare settings.

**Results**

**Demographics and Patient Characteristics**

The mean age of the patients was 41.80 years (SD ± 18.87 years; range 6–84 years), with 21 (42\%) males and 29 (58\%) females and from them 34/50 (68\%) patients were in the adult age group (25–64 years). Unformed stool passages ranged from 3–5 to > 10 per day; and 11/50 (22\%) of the patients had more than 8 passages per day. A history of hospitalization showed that 8/50 (16\%) patients had a hospital stay in the last three months before enrollment in the study. Among all patients, 27/50 (54\%) had hospital-acquired CDI (HA-CDI) and 23/50 (46\%) had community-acquired CDI (CA-CDI). Nineteen (38\%) patients were hospitalized in gastroenterology, followed by oncology (16\%), and internal (10\%) as the most common hospital wards. Fifteen (30\%) patients had a history of inflammatory bowel disease (IBD) and 11/50 (22\%) patients suffered from diarrhea at the time of admission. The average length of time before diarrhea was 73.64 hours (range 5-365 h). Thirty-nine (78\%) patients had a previous history of antibiotic use before hospital admission. The most common antibiotics used were metronidazole (27/50, 54\%) followed by ciprofloxacin (11/50, 22\%), carbapenem class (9/50, 18\%), vancomycin (8/50, 16\%), and extended-spectrum cephalosporins (6/50, 12\%). Moreover, the usage of antacid and
immunosuppressive drugs was observed in 10/50 (20%) and 11/50 (22%) of patients, respectively. The demographic data and clinical characteristics of CDI patients are presented in Supplementary Table S2.

**Ce-pcr Ribotyping**

Among 50 *C. difficile tcdA+B* isolates, 17 distinct RTs were identified. The most common RTs were RT 001 (13/50, 26%) and RT 126 (10/50, 20%), followed by RT 014, RT 005, and RT 070 each with three isolates (6%). The remaining RTs included one or two isolates. Three isolates had no profiling match with the WEBRIBO and remained unrecognized.

**Paloc Genes Detection**

The presence of the *tcdR* and *tcdE* genes was detected in all *tcdA+B* isolates in the study (n = 50). The simultaneous presence of the CDT genes, *cdtA* and *cdtB*, was found in 12/50 (24%) of isolates.

**tcdC Genotypes**

Of the 50 isolates investigated, 15 (30%) had no deletion in the *tcdC* sequence; they had a wild-type *tcdC* genotype. Thirty-five (70%) isolates had genetic variations in the *tcdC* gene compared to the *tcdC* gene of the VPI 10463 strain as a reference sequence. The complete amino acid sequence alignment of TcdC from 50 *tcdA+B* isolates is presented in Figure S1. Twelve isolates (24%) possessed a G to T transition at nucleotide 148 and belonged to the *tcdC-sc3* genotype. Eleven isolates (22%) had 39 bp deletion and also a C to T transition at nucleotide 184 and were assigned as the *tcdC-A* genotype. The C184T leads to a nonsense mutation and resulted in a truncated TcdC protein. Nine isolates (18%) had a G to T transition at nucleotide 21 and belonged to the *tcdC-sc9* genotype. In addition, the remaining three isolates were assigned to *tcdC-B*, *tcdC-sc14*, and *tcdC-sc15* genotypes. The majority of the wild-type genotypes (93.3%) and 77.8%, 75% and 18.2% of *tcdC-sc9*, *tcdC-sc3* and *tcdC-A* variants, respectively, were negative for *cdtAB* genes. The remaining genotypes including *tcdC-B*, *tcdC-sc14*, and *tcdC-sc15* did not harbor the *cdtAB* genes. The characteristics of various *tcdC* genotypes identified in this study are presented in Table 1.
Table 1
Characteristics of the various tcdC genotypes identified in tcdA⁺B⁺ isolates in this study.

<table>
<thead>
<tr>
<th>tcdC genotype</th>
<th>Mutations (nucleotide position)</th>
<th>Number of isolates (%)</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdC-sc9</td>
<td>G21T, insertion of A (443), G452A, insertion of GT (454–455), T456A</td>
<td>9 (18)</td>
<td>MN548810, MN548811, MN548812, MN548813, MN548814, MN548815, MN548816, MN548817, MN548818</td>
</tr>
<tr>
<td>tcdC-sc3</td>
<td>G148T, insertion of A (443), G452A, insertion of GT (454–455), T456A</td>
<td>12 (24)</td>
<td>MN548798, MN548799, MN548800, MN548801, MN548802, MN548803, MN548804, MN548805, MN548806, MN548807, MN548808, MN548809</td>
</tr>
<tr>
<td>tcdC-sc15</td>
<td>A117G, C183T, C363T, insertion of A (443), G452A, insertion GT (454–455), T456A</td>
<td>1 (2)</td>
<td>MN548820</td>
</tr>
<tr>
<td>tcdC-B</td>
<td>18 bp deletions (330–347), insertion of A (443), G452A, insertion GT (454–455), T456A</td>
<td>1 (2)</td>
<td>MN548797</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>15 (30)</td>
<td>MN548821, MN548822, MN548823, MN548824, MN548825, MN548826, MN548827, MN548828, MN548829, MN548830, MN548831, MN548832, MN548833, MN548834, MT040191</td>
</tr>
</tbody>
</table>

Distribution of Toxinotypes and RTs with Relation to tcdC Genotypes

Among the tcdA⁺B⁺ isolates 5 different toxinotypes were identified (Table 2). Toxinotype 0 (nonvariant) was the most common toxinotype (33/50, 66%) identified within the isolates, followed by toxinotypes V (11/50, 22%), XIII (4/50, 8%), XXVIII (1/50, 2%) and 0/v (1/50, 2%). Isolates with toxinotype 0 were classified in 13 RTs and 12/33 (36.4%) of these isolates had RT/toxinotype 001/0. The majority of isolates of toxinotype V belonged to ribotype 126 (81.8%), other ribotyping profiles identified were RTs
038 and WRT628. The distribution of RTs in various toxinotypes showed that the RT 001 included 001/0 (12/13, 92.3%) and 001/XIII (1/13, 7.7%). Moreover, RT 126 contained 126/V (9/10, 90%) and 126/XXVII (1/10, 10%). Isolates with toxinotype 0 were mostly recognized as \textit{tcdC} wild-type genotype (13/33, 39.4%), followed by \textit{tcdC-sc3} (9/33, 27.3%), \textit{tcdC-sc9} (6/33, 18.2%), \textit{tcdC-A} (2/33, 6%), \textit{tcdC-B} (1/33, 3%), \textit{tcdC-sc14} (1/33, 3%) and \textit{tcdC-sc14} (1/33, 3%). Toxinotype V was classified in \textit{tcdC-A} (8/11, 72.7%), wild-type (2/11, 18.2%) and \textit{tcdC-sc3} (1/11, 9.1%) genotypes. The distribution of toxinotypes and RTs in relation to \textit{tcdC} genotypes are presented in Table 2.
Table 2
The distribution of toxinotypes and RTs in relation to tcdC genotypes among tcdA⁺B⁺ isolates.

<table>
<thead>
<tr>
<th>Toxinotype</th>
<th>Ribotype</th>
<th>tcdC genotype</th>
<th>CDT genes</th>
<th>Number of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>126</td>
<td>tcdC-A</td>
<td>cdtA⁺B⁺</td>
<td>6 (12)</td>
</tr>
<tr>
<td>0</td>
<td>139</td>
<td>tcdC-sc9</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>029</td>
<td>wild-type</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0</td>
<td>085</td>
<td>tcdC-sc3</td>
<td>cdtA⁺B⁺</td>
<td>1 (2)</td>
</tr>
<tr>
<td>V</td>
<td>WRT628</td>
<td>wild-type</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>Unrecognized</td>
<td>tcdC-sc3</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0</td>
<td>001</td>
<td>tcdC-sc3</td>
<td>Negative</td>
<td>5 (10)</td>
</tr>
<tr>
<td>XXVIII</td>
<td>126</td>
<td>tcdC-A</td>
<td>cdtA⁺B⁺</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>001</td>
<td>tcdC-sc14</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>039</td>
<td>tcdC-sc15</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>070</td>
<td>tcdC-sc9</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0/v</td>
<td>038</td>
<td>tcdC-sc3</td>
<td>cdtA⁺B⁺</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>015</td>
<td>wild-type</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>Unrecognized</td>
<td>wild-type</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>014</td>
<td>tcdC-sc9</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0</td>
<td>103</td>
<td>tcdC-sc3</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0</td>
<td>001</td>
<td>wild-type</td>
<td>Negative</td>
<td>5 (10)</td>
</tr>
<tr>
<td>0</td>
<td>019</td>
<td>tcdC-B</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>V</td>
<td>126</td>
<td>tcdC-A</td>
<td>cdtA⁺B⁺</td>
<td>2 (4)</td>
</tr>
<tr>
<td>0</td>
<td>005</td>
<td>wild-type</td>
<td>Negative</td>
<td>2 (4)</td>
</tr>
<tr>
<td>V</td>
<td>038</td>
<td>wild-type</td>
<td>cdtA⁺B⁺</td>
<td>1 (2)</td>
</tr>
<tr>
<td>XIII</td>
<td>005</td>
<td>tcdC-sc9</td>
<td>cdtA + B⁺</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>014</td>
<td>tcdC-A</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>001</td>
<td>tcdC-A</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>004</td>
<td>tcdC-sc9</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>
### Discussion

There is an ongoing debate in the literature about the increasing trend of CDI worldwide [25, 26]. The emergence of multidrug resistant, and so-called hypervirulent RTs, has undoubtedly contributed to the rapid increase in the number of CDI cases [16, 27]. Studies that included ribotyping for the characterization of causative *C. difficile* isolates, reported a different prevalence for the most frequent RTs in individual countries and time spans [28]. Molecular fingerprinting and surveillance studies of CDI are recommended in order to monitor its epidemiological variations [29]. Currently, there are limited data on toxinotypes and *tcdC* sequence variations among *tcdA*/*B*+ *C. difficile* isolates from Iranian patients. PaLoc shows a mosaic structure in *C. difficile* isolates, and the occurrence of genetic mutations in this locus is essential for the generation of toxinotype variants [5, 30, 31]. In this study, we showed that toxinotypes 0 and V were the most frequent types among *tcdA*/*B*+ isolates. The PCR ribotypes of isolates within toxinotype 0 were classified into 13 RTs and largely belonged to 001/0, whereas the majority of toxinotype V isolates consisted of the 126/V type. Our findings resemble a recent study from Iran, in which the toxinotypes 0 (81.57%) and V (18.42%) were reported as the most common toxinotypes [32]. Moreover, another study from diarrheic individuals in an Iranian hospital reported toxinotypes 0 and V as the most prevalent types [33]. Based on these results, PaLoc shows slight genetic changes in the predominant toxinotypes, 0 and V, from Iranian isolates. However, other studies from Asian countries reported type 017/VIII as the most frequent variant toxinotype [24, 34]. But, in a study from Kuwait, which is a neighboring country, 71.4%, 19% and 9.4% of *C. difficile* isolates were assigned to 0, V-like and XII toxinotypes, respectively [35]. In addition, another study at a tertiary care center in Lebanon reported 80.8% of isolates belonged to a toxinotype 0-like [36]. In contrast, 078/V (8%), 027/III (5%), 017/VIII (4%), 126/V (3%), and 023/IV (3%) were the most RT/toxinotype patterns among 395 isolates from 73 hospitals and 26 countries in Europe, while 027/III and 078/V are often isolated in the United States [24, 37, 38].

Our results suggest that CE ribotyping could be utilized for the further distinction of *tcdA*/*B*+ isolates with identical toxinotypes. Accordingly, isolates with toxinotypes 0, XIII and V were distributed in thirteen, four and three RTs, respectively. Furthermore, it is worth to considering the variability in the *tcdA* and *tcdB* when laboratory diagnostic assay is designed [24]. It is assumed that the different RTs within a certain toxinotype may have evolved from a common ancestral strain. This event could be due to independent

<table>
<thead>
<tr>
<th>Toxinotype</th>
<th>Ribotype</th>
<th><em>tcdC</em> genotype</th>
<th>CDT genes</th>
<th>Number of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIII</td>
<td>070</td>
<td><em>tcdC</em>-sc9</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>XIII</td>
<td>001</td>
<td><em>tcdC</em>-sc3</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>0</td>
<td>003</td>
<td>Wild-type</td>
<td>Negative</td>
<td>1 (2)</td>
</tr>
<tr>
<td>XIII</td>
<td>405</td>
<td><em>tcdC</em>-sc9</td>
<td><em>cdtA</em>+/B+</td>
<td>1 (2)</td>
</tr>
<tr>
<td>V</td>
<td>126</td>
<td><em>tcdC</em>-sc3</td>
<td><em>cdtA</em>+/B+</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>
mutations or horizontal gene transfer (HGT) that occurred in the PaLoc of an ancestral strain [4]. In a previous study, a sequence analysis of the 16S rRNA gene revealed that the strains with toxinotypes V, VI, and XI had a single base change when compared to the VPI 10463 strain [30].

In our previous study that used a large collection of *C. difficile* isolates, RT 001 (12.9%), RT 126 (11.2%), and RT 084 (3.3%) were the most frequent RTs identified in CDI patients across different hospitals and medical centers in Tehran [39]. In a recent study by Baghani et al., RT 001 (32.3%) and RT 126 (9.2%) were also reported as the most common RTs isolated from patients with CDI in Tehran [40]. To date, RT 027 isolates have not been reported from Iranian patients. Similarly, no isolate of RT 027 was recognized in the present study. A prominent characteristic of the RT 001 and RT 027 is their multiple resistance to antimicrobials such as erythromycin and moxifloxacin [41]. Previous studies also identified 126/V among different animals, and it was frequently detected within piglet and calf isolates [42–44]. Interestingly, some of our RTs including 001, 003, 005, 014, 015, 029, 038, 039, 070, 103, and 126 had been previously identified from livestock [44–48]. Although little is known about the animal-associated PCR RTs of *C. difficile* within animal hosts or livestock in Iran, animal sources can be regarded as a potential reservoir for zoonotic transmission of this opportunistic pathogen.

Sequence analysis of tcdC, predicted that six variants (35/50, 70%) were present in our studied isolates. Recently, Aliramezani et al. identified two tcdC variants out of 38 isolates including tcdC-sc3 (44.73%) and tcdC-A (18.43%) genotypes [32]. In that study, 36.84% of the isolates were identified as a wild-type tcdC genotype which is similar to our findings. In another study performed at two hospitals in Canada, of the 214 CDI cases that were genotyped over one year, 51.9% were caused by tcdC variants [49]. Furthermore, the majority of the tcdC-A variants belonged to RTs 001, 126 and toxinotype V in this study. However, there are still conflicting data on the functional role of TcdC in toxin production. A modulatory role for TcdC in regulating toxin expression has been suggested as a minor determinant of the (hyper)virulence of *C. difficile* [16, 43, 44, 50]. On the other hand, Stewart et al. proposed that the risk of rCDI was strongly increased and predicted by the existence of the cdt genes and specific tcdC single nucleotide polymorphisms (SNPs) C184T and A117T, which introduced premature stop codons that resulted in significant protein truncation [51]. Our findings suggest that further studies are needed, not only to understand the possible impact of accumulated genetic mutations but also changes to the tcdC that may allow epidemic *C. difficile* isolates to become the predominant hypervirulent isolates worldwide. However, based on these preliminary data, a comprehensive and definite statement regarding the molecular fingerprinting of tcdA+B+ *C. difficile* clinical isolates may not be made and underlines one of the limitations of the current study. Further investigations, including whole genome sequencing, are needed for a deeper understanding of the spread of *C. difficile* lineages.

**Conclusions**

The present study demonstrates that the majority of clinical tcdA+B+ isolates of *C. difficile* frequently harbor tcdC genetic variants. We also found that RT 001/0 and RT 126/V are the most common
RTs/toxinotypes among those isolates from Iran. Further studies are required to investigate the putative association of various tcdC genotypes with CDI severity and its recurrence.

Methods

C. difficile isolates and data collection

A total of 50 C. difficile isolates with tcdA⁺B⁺ genotype were included in this study. Patient demographic data, antibiotic and medication history and clinical details were recorded for all subjects.

C. difficile culture and DNA extraction

All isolates were retrieved from storage by subculture on cycloserine-cefoxitin-fructose agar (CCFA, Mast) supplemented with 7% horse blood under anaerobic conditions of 85% N₂, 10% CO₂ and 5% H₂ (Anoxomat® Gas Exchange System, Mart Microbiology BV) at 37 °C for 48–72 h as previously described [52, 53]. Briefly, 3–5 fresh colonies were picked from the plates, suspended in 1 ml of molecular biology-grade water, and then the genomic DNA was extracted using the InstaGene Matrix kit (Bio-Rad, USA) according to the manufacturer’s instructions. An assessment of the concentration and purity of the extracted DNAs was determined by NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). DNA samples were stored at -20 °C until use.

Molecular Identification And Detection Of Toxin Genes

A multiplex PCR for the detection of tcdA, tcdB, cdtA, cdtB, and 16S rDNA genes was carried out as previously described [54]. The amplification of tcdE, tcdR, cdu2, and cdd3 genes was performed in another multiplex PCR format as previously described [55]. The oligonucleotide primers and amplicon size of each target gene are indicated in Supplementary Table S1.

The tcdC gene sequencing

The sequence of complete tcdC gene was amplified using specific primers C1 and C2 by PCR as previously described [56]. The reaction mixture contained 12.5 µl of Taq DNA Polymerase Master Mix (Ampliqon, Denmark), 1 µl (10 pM/µl) of each primer, 8.5 µl of distilled water, and 2 µl (100 ng) of DNA template in a final volume of 25 µl. Amplifications were carried out using a thermocycler (Eppendorf, Hamburg, Germany) under the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. PCR products were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific, Fermentas, USA). Sequencing was performed using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were edited by Chromas Lite version 2.5.1 (Technelysium Pty Ltd., Australia) software. The edited nucleotide sequences of tcdC were subjected to inframe translation using BioEdit version 7.2.5 (Hall, 1999), and were aligned to the tcdC sequence gene...
of *C. difficile* strain VPI10463 (accession number: X92982.1). In addition, all DNA sequences were compared with those existing in the NCBI database and deposited in the GenBank (www.ncbi.nlm.nih.gov).

**Toxinotyping By Pcr-restriction Fragment Length Polymorphism (rflp)**

A PCR-RFLP based toxinotyping scheme was performed as previously described [57]. Briefly, all *tcdA*+*B*+ isolates were subjected to toxinotyping using specific primers for B1 and A3 fragments and subsequent digestion with restriction enzymes *Hinc*II, *Acc*I and *Eco*RI (Roche, Germany). The toxinotype of each isolate was determined according to the combination of B1 and A3 digest patterns.

**Capillary Electrophoresis Ribotyping**

A capillary electrophoresis (CE) PCR ribotyping was conducted at the Department of Medical Microbiology, Motol University Hospital, Prague, Czech Republic according to the consensus PCR ribotyping protocol [58]. The CE ribotyping profiles were compared with the WEBRIBO database [59].

**Statistical analysis**

Data analyses were carried out using SPSS (version 23, IBM Corp.). Descriptive results were demonstrated as frequencies and percentages.

**Declarations**

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**Author contributions**

M. Kodori and M. Azimirad: microbiological experiments, molecular tests, data collection; M. Krutova: CE-PCR ribotyping; A. Yadegar: design of the study, methodology, conceptualization; A. Yadegar, G. Eslami and Z. Ghalavand: project administration; M. Kodori and A. Yadegar: drafting and editing of the manuscript; A. Abadi: statistical analysis; M.R. Zali: critical manuscript revision. All authors approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information Figure S1.

Ethics approval and consent to participate

This study was undertaken at the Department of Anaerobic Bacteriology in Research Institute for Gastroenterology and Liver Diseases (RIGLD) in Tehran, Iran. It was granted with ethical approval from the Ethical Review Committee of Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.MSPREC.1398.736). Isolates of *C. difficile* were given numerical codes to anonymize the patient's identity.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interest.

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