ORIGINAL ARTICLE

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

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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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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).