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RESEARCH ARTICLE

Isolation and characterization of a multidrug-resistant *Clostridioides difficile* toxinotype V from municipal wastewater treatment plant

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Abstract

Purpose Wastewater treatment plant (WWTP) is regarded as a potential source for transmission of *Clostridioides difficile* from urban areas into the surface water, through feces of human and animals. The aim of this study was to screen and characterize the *C. difficile* bacteria in inlet and outlet wastewater of different WWTPs in Tehran, Iran.

Methods Totally, 72 samples were collected from three different WWTPs (inlet site and outlet sites) during a year. *C. difficile* was isolated and characterized in terms of toxins, toxinotype, resistance profile and genes, and colonization factors using PCR.

Results One *C. difficile* toxinotype V was isolated from the outlet samples. The isolate was susceptible to vancomycin but resistant to metronidazole, tetracycline, ciprofloxacin, and moxifloxacin using MIC Test Strips. The isolated *C. difficile* was toxigenic (*tcdA*, *tcdB*, *cdtA*, *cdtB* positive and CPE positive) and had *tcdC-A* genotype. No mutations were found in *fliC* and *fliD*. The *slpA* sequence type was 078 − 01. The *C. difficile* was positive for *tetM*, *int*, but negative for *vanA*, *nim*, and *tndX* genes. Mutations were not observed in *gyrA* and *gyrB* genes.

Conclusions This study provided evidence of presence of a multidrug-resistant *C. difficile* toxinotype V in one of the municipal WWTP. The transmission of such isolate to the environment and reuse of treated wastewater by human pose a threat to human health and dissemination of antibiotic resistant bacteria which are untreatable.

Keywords *Clostridioides difficile* · Multidrug-resistance · Toxinotype V · Wastewater treatment plant

Introduction

Clostridioides difficile is a cause of hospital-associated *C. difficile* infection (HA-CDI) or community- associated *C. difficile* infection (CA-CDI). In recent years, more than 25% of CDI cases have been attributed to the community source [1]. CA-CDI is diagnosed in individuals without any risk factors for CDI and contact with health care settings. In addition to asymptomatic carriers, environment, animals, and food are implicated in transmission of CA-CDI to human [2]. Wastewater treatment plant (WWTP) is regarded as an important potential source for transmission of enteric pathogens

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such as *C. difficile*, and viruses from urban areas into the surface water through feces of human and animals [3]. As hotspots that contain a wide variety of resistant bacteria, WWTPs play an important role in dissemination of antibiotic resistance genes (ARGs) via transposons or other mobile genetic elements (MGEs) among bacteria [3]. Characterization of resistant pathogen bacteria (such as *C. difficile* toxinotype V) in the inlet and outlet of WWTPs as hotspots for exchange of antibiotic resistant genes are of highly importance [4]. Release of treated wastewater to environments can lead to the discharge of resistant pathogens and ARGs. It was demonstrated that ARGs are not reduced in effluents compared with influents of WWTPs, and indeed they are enriched in the effluent [4].

In the last decades, metronidazole and vancomycin are used for treatment of CDI. The genes encoding resistance to metronidazole (*nim*) and vancomycin (*vanA*) are carried on plasmid or chromosome and transposon 1546 (Tn*1546*), respectively [5, 6]. The *nim* and *vanA* genes were previously detected in wastewater and drinking water [5, 6]. The hypervirulent strains of *C. difficile* PCR-ribotype 078 (RT078 or toxinotype V) and RT027 are resistant to tetracycline [7] and fluoroquinolones, respectively, [8] that is an important concern at treatment. The resistance to fluoroquinolones is due to mutations in *gyrA* and *gyrB* genes and resistance to tetracycline is due to *tetM* gene [7, 8].

Outbreaks of CDI have been associated with the emergence of hypervirulent strains; RT078 (toxinotype V) and RT027 (toxinotype III) with increased mortality in Europe, North America, and Asia [9]. The hypervirulent strains produce higher amounts of toxins such as TcdA (enterotoxin), TcdB (cytotoxin), and CdtA/B and have mutations in a putative negative regulator (TcdC) of toxins. Also, these strains are resistant to fluoroquinolones [10]. The colonization factors such as *slpA* (surface layer protein), *fliC* (flagellum fragment), *filD* (flagellum cap), *cwp*84, and *cwp*66 (cell wall proteins) are also implicated in colonization, survival and pathogenicity of *C. difficile* strains [11].

Considering the mentioned points, effluents of WWTP have to be examined for contamination by harmful microbes such as *E. coli* and Enterococci as microbial indicators [12]. Presence of *C. difficile* as a cholrine- rellisient spore-forming bacterium has been rarely investigated in the contaminated aquatic environments such as wastewaters [2, 9, 13–18]. The possible contamination of WWTPs outlet with pathogenic bacteria including multidrug-resistant *C. difficile* toxinotype V can play a role in the development of severe CDI and cause serious health problem by releasing the wastewater into the environment, and reuse of treated wastewater by human [14].

In this study, the inlet and outlet wastewater of three different municipal WWTPs in Tehran, Iran, was examined for *C. difficile* existence in a year. Moreover, toxins, colonization factors, resistance genes, and toxinotype of isolated *C. difficile* were identified.

Materials and methods

Sampling scheme and sample collection

A cross-sectional study was performed from inlets and outlets of three municipal WWTPs which were sampled in Tehran during a year on a monthly basis, between June 2016 and 2017. The location of each WWTP in Tehran, capacity (m^3/d), and population equivalent are indicated in Table 1. Totally, 72 samples were collected from three different WWTPs. The wastewater samples were taken in sterile bottles during 12 sampling visits for each WWTP. Sampling points were similar for three WWTPs. In each visit, two samples of wastewater were taken from each point (site) including inlets site before initial screening (untreated) and outlets at the point of release (treated). Each sample was collected in 500-ml sterile bottles. The collected samples were transported on ice to laboratory and preserved at 4 °C for a maximum of 1 day to be tested.

Isolation of *C. difficile*

For isolation of *C. difficile*, 100 ml of non-concentrated outlet wastewater sample (1:1 and 1:10 dilutions in sterile physiological saline) and 100 ml of non-concentrated inlet wastewater sample (1:100 and 1:1000 dilutions in sterile physiological saline) were concentrated using the filtration method through a cellulose membrane filter (FILTEP-BIO) with a 0.45 μm pore size using a vacuum pump (Membran-Vakuumpumpe, Germany). The filters were then enriched in 25 ml of *Clostridium difficile* Moxalactam Norfloxacin (CDMN) broth with 0.1% sodium taurocholate and incubated in an anaerobic atmosphere using the Anoxomat jar system (MART Microbiology B.V., the Netherlands) at 37 °C for 7–10 days [2]. After incubation period, 2 ml of the enriched broth culture (sediment) was added to 2 ml of absolute ethanol and incubated for 1 hour at room temperature and then centrifuged at 4,000 rpm for 10 min. The supernatant was discarded and the pellet (100 μl) was streaked on CDMN agar and incubated anaerobically at 37 °C for 48–72 hours. After incubation, the plates were checked for *C. difficile* colonies odor and morphology. The suspected colonies with raised, grey-white and opaque appearance were Gram-stained. Single colonies with Gram-positive bacilli were sub-cultured onto Brucella Agar supplemented with 5% sheep blood (BBA) plates and incubated anaerobically for 48 hours at 37 °C. Then, colonies were examined for L-proline aminopeptidase (C. diff PRO™ Kit) [15]. Number of colonies as colony forming units (CFU) per

Table 1 Description of wastewater treatment plants

WWTP no.	Location in Tehran	Capacity (m^3/d)	Population equivalent (PE) (Person)
1	East	600	7000
2	West	24,000	100,000
3	South	450,000	4,200,000

100 µl was recorded. In order to examine the toxicity of the isolate and evaluate the cytopathic effect (CPE), the cytotoxicity assay was performed [19].

Antimicrobial susceptibility testing (AST)

AST was performed using MIC Test Strips (Liofelichem, Italy) for vancomycin, metronidazole, tetracycline, and fluoroquinolones (ciprofloxacin and moxifloxacin) [20, 21]. Concentration gradient of vancomycin, metronidazole, and tetracycline ranged from 0.016 to 256 mg/L and of ciprofloxacin and moxifloxacin ranged from 0.002 to 32 mg/L. Fresh colonies (24-hour) were used for AST to prepare a suspension with 1 McFarland turbidity standard. The plates were then incubated in anaerobically at 37 °C for 48 hours. They were checked at 24 hours and 48 hours.

Molecular characterization of *C. difficile*

DNA extraction was performed using Chelex100 (Sigma, USA) on *C. difficile* grown on Brucella Agar supplemented with 5% sheep blood [22]. Conserved genes including *tpi*, *gluD*, *cdd*3, *cdu*2, and 16S *rDNA* were amplified as described previously in order to identify the *C. difficile* [23–26]. The identified *C. difficile* was examined for the presence of *tcdA*, *tcdB*, *cdtA/B* and *tcdC* genes as previously described [23, 27] and subsequently the *tcdC* PCR product was subjected to sequencing. Also, *slpA*, *fliC*, and *fliD* were amplified and sequenced for *C. difficile* as described previously [28–30]. The isolate was examined for the presence of *vanA*, *nim*, *tetM*, *gyrA*, and *gyrB* genes using PCR [31–34]. The genes *int* and *tndX* were also amplified as indicators of Tn*916*-like and Tn*5397*-like, respectively [35, 36]. Toxinotyping was performed according to the method described by Rupnik et al., [37].

Results

Isolates

In the present study, three isolates were detected in inlet of WWTPs located in East, West, and South using PCRs targeting *gluD* and *tpi* genes, but these isolates did not generate amplicons for the other conserved genes. Out of 72 samples, one sample (1.38%) was found to contain *C. difficile* by a combination of tests as Gram staining (Gram-positive bacillus), L-prolin aminopeptidase-positive but negative indole-negative reactions and PCR for five conserved genes. This isolate was recovered from outlet of WWTP located in west of Tehran. The number of colonies was 60 CFU per 100 µl of cultured suspension on BBA.

Antibiotic susceptibilities and antibiotic-resistant genes

The Minimum inhibitory concentration (MIC) (mg/L) using MIC Test Strips for vancomycin, metronidazole, tetracycline, ciprofloxacin, and moxifloxacin are shown in Fig. 1. With respect to the mentioned resistance genes, the *C. difficile* isolate was positive for some of them (Fig. 2). The characteristics of antibiotic resistance in *C. difficile* isolate are indicated in Table 2.

Molecular characteristics

The *C. difficile* isolate contained *tcdA*, *tcdB* and, *cdtA*/*B* genes and yielded 100% CPE. The toxigenic *C. difficile* belonged to toxinotype V. The genotype of *tcdC* was *tcdC-A* with 11 point mutation and one 39 bp deletion between 341 and 379 nucleotides, resulted in severe truncation of the TcdC protein. The sequence of *tcdC* (718 bp) for the isolate from outlet is available in the Supplementary file. Using *slpA* sequence typing, this isolate was typed as 078 − 01. Sequencing of *fliC* and *fliD* genes of this isolate revealed that they had no mutation and were identical to *fliC* and *fliD* genes of the *C. difficile* 630. The nucleotide sequences of *fliC*, *fliD slpA*, and *tcdC* genes were deposited in GenBank. The sequence length of *fliC*, *fliD*, and *slpA* genes along with the Genbank accession numbers are shown in Table 3.

The mutations of quinolone-resistance determining region (QRDR) *gyrA* and *gyrB* genes leading to amino acid substitutions in Thr82 → Ile and Thr82 → Val of GyrA and Ser366 → Val, Ser416 → Ala, Ser366 → Ala, Asp426 → Asn, and Asp426 → Val of GyrB, were not found in the isolate. The nucleotide sequences of *gyrA* and *gyrB* genes were deposited

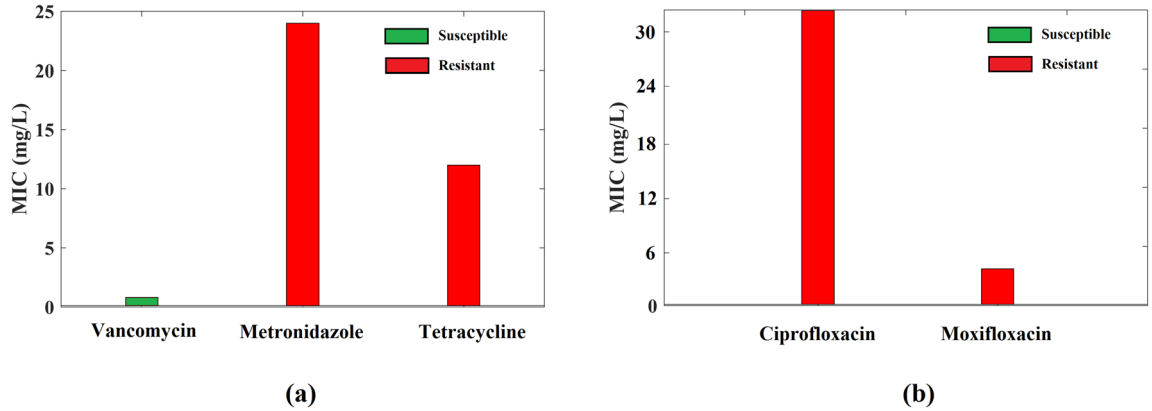

Fig. 1 Minimum inhibitory concentration (MIC; mg/L) values of different antibiotics against the isolated *C. difficile*. (**a**) Concentration gradient of vancomycin, metronidazole, and tetracycline ranged from 0.016 to 256 mg/L. The MIC>2 mg/L for vancomycin and metronidazole and MIC > 0.25 mg/L for tetracycline are considered resistant (red color). The MIC ≤ 2 mg/L is considered susceptible (green color). (**b**) Concentration gradient of ciprofloxacin and moxifloxacin ranged from 0.002 to 32 mg/L. The MIC>4 mg/L for ciprofloxacin and moxifloxacin are considered resistant (red color)

in GenBank under the accession numbers of MH753335 and MH753336, respectively.

Discussion

Only one *C. difficile* isolate was recovered from three different municipal WWTPs in one year between June 2016 and June 2017. The Abiotic parameters such as the competition among different species of bacteria and also the oxygen concentration might be responsible for the observed differences in isolation rate of *C. difficile* from the WWTPs inlet vs.. the outlet [38]. The study conducted by Nikaeen et al.., in central Iran; Isfahan reported no *C. difficile* in inlet and outlet samples or in raw sludge of activated sludge similar to our results [2]. The higher rate of *C. difficile* (13.6%) was observed in the wastewater samples than we found but from the other samples in WWTP (digested sludge). The discrepancy in the isolation rate of *C. difficile* might be partly related to the selected method or sampling sites of WWTPs; for instance we did the sampling from the inlet and outlet of WWTPs whereas they took the samples from several sites including raw sludge, digested sludge, dewatered digested sludge, inlet of anaerobic pond, outlet of anaerobic pond, outlet of facultative pond and outlet of maturation pond. However, there are a few studies all over the world that have focused on isolation of *C. difficile* from WWTPs [2, 9, 13–18]. The incidence of *C. difficile* in wastewater samples in different countries is very variable (Fig. 3). The recovery of *C. difficile* from our samples was lower compared to other studies, which could be due to the difference in detection methods, selection of culture medium and/or sampling sites. The differences in isolation rates of multiple studies indicate that it is better to have a universal isolation method for *C. difficile* detection from wastewater. There are reports from Italy, France, and Australia about the environmental dispersion of Clostridiaceae via outlet of WWTPs between 2008 and 2010. These reports have focused on ability of spore detachment from flocks of sludge into the outlet wastewaters [39–41]. In our study, release of *C. difficile* into the outlet of WWTP was observed that may provide a route for *C. difficile* dissemination from WWTP to environment. It has been speculated that *C. difficile* strains or their spores, due to long-term persistence can spread into the environment. In reality, it is likely that *C. difficile* is a waterborne pathogen that can lead to CDI in human when the contaminated water is consumed [7]. In other words, the *C. difficile* spore formation (even in very low levels [42]) provides a potential for wide dissemination and contamination of environment through wastewater that are considered as an important transmission routes of CDI [14].

In the current study, the *C. difficile* isolate from the outlet was considered toxigenic according to positive results for PCR of *tcdA*, *tcdB*, and *cdtA/B* genes and observation of a 100% CPE on Vero cells after 24 hours. This finding is consistent with the other studies showing the presence of toxigenic *C. difficile* in aquatic environments [2, 43]. In another study, Saif et al., isolated the *C. difficile* from treated water samples in South Wales and reported that 84.6% of isolates were TcdA positive [16]. The toxigenic *C. difficile* is associated to a spectrum of CDI ranging from asymptomatic colonization, self-limiting and mild disease to severe one including pseudomembranous colitis that is life-threatening, toxic megacolon, sepsis and death [44]. The *tcdC* sequencing is one of the indicators of the hypervirulent *C. difficile* strains [45]. The sequence of *tcdC* gene obtained from our isolate illustrated that the genotype was *tcdC-A*. Genotype *tcdC-A* was observed in toxinotypes V and VI of *C. difficile* [46].

It was found that the isolated *C. difficile* in this study from the outlet of a WWTP is multidrug-resistant (MDR) strain. It was susceptible to vancomycin with the MIC of lower than the

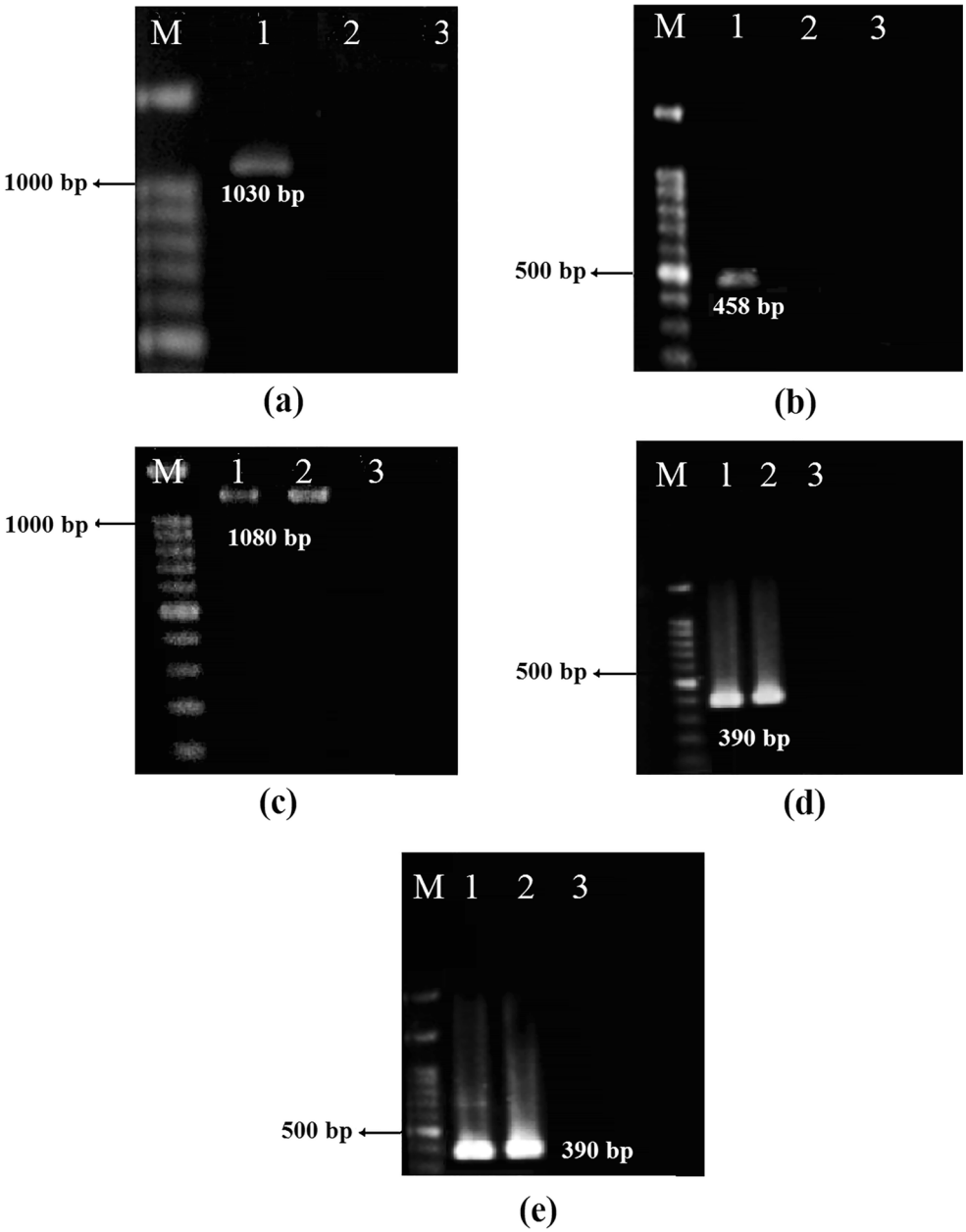

Fig. 2 Agarose gel electrophoresis of PCR products for antibiotic resistance genes in the *Clostridium difficile* isolate. Lanes 1 to 3 show positive control, *Clostridium difficile* isolate from WWTP, and negative control, respectively. M lane (DNA Ladder) depicts 100 base pairs Ladder. (**a**) The isolate was negative for *vanA* gene (1030 bp); (**b**) The isolate was negative for *nim* gene (458 bp); (**c**) The isolate was positive for *tetM* gene (1080 bp); (**d**) The isolate was positive for *gyrA* gene (390 bp) and (**e**) The isolate was positive for *gyrB* gene (390 bp)

Table 2 Characteristics of antibiotic resistance in *Clostriudm difficile* isolate

Antibiotic	Targt gene	Sequence Length (bp)	Resistant or Susceptible using MIC Test Strips
Vancomycin	*vanA*	Not amplified*	Susceptible
Metronidazole	*nim*	Not amplified*	Resistant
Tetracycline	*tetM*	1080	Resistant
	int (indicator of Tn*916*-like)	925	
	tndX (indicator of Tn*5397*-like)	1602	
Ciprofloxacin	*gyrA* and *gyrB*	390	Resistant
Moxifloxacin	*gyrA* and *gyrB*	390	Resistant

* Not amplified: PCR was done but the relevant fragment was not observed

Table 3 The sequence length of *fliC*, *fliD*, and *slpA* genes along with the GenBank accession numbers

Gene	Associated protein	Sequence length (bp)	GenBank accession numbers
filC	flagellum fragment	838	MH885485
fliD	flagellum cap	1425	MH698447
SlpA	Surface-layer protein	1005	MH473346

breakpoint (2 mg/L), and was resistant to metronidazole, fluoroquinolones, and tetracycline (Fig. 1). The resistance pattern of this isolate was similar to that of clinical isolates recovered from patients suffered from CDI in Iran [47]. The presence of *C. difficile* strains which exhibited high-level multi-resistance to erythromycin, tetracycline, clindamycin, and moxifloxacin and carried several ARGs including *ermB*, *fusA* and *tetM* have been detected in surface waters [48]. The *vanA* and *nim* genes were not found in the *C. difficile* isolate (Fig. 2). Another ARG, *tetM* gene, was detected in the *C. difficile* isolate and the possible transposon of *tetM*, Tn*916*-like, was also detected using *int* gene PCR amplification (Table 2). More than 75% of *C. difficile* RT078 isolates (toxinotype V) were known as tetracycline-resistant in North American and European countries [7]. With regard to fluoroquinolones, several studies demonstrated that there is an association between nucleotide substitutions of *gyrA* and *gyrB* genes and resistance to fluoroquinolones [34, 49], However, such nucleotide substitutions were not found in the *C. difficile* isolate in this study. Since the *C. difficile* isolate was resistant to fluoroquinolones, this finding indicates that the other mechanisms may play a role in the resistance to fluoroquinolone.

The colonization factors including SlpA, FliC, and FliD proteins play a role in attachment of *C. difficile* to the intestine mucus layer [50]. The sequencing of *slpA* has been employed to type *C. difficile* strains [51]. The *slp*AST (*slpA* sequence type) of the isolate was 078 − 01. This type of *slpA* was previously observed in *C. difficile* RT078 with GenBank accession no. AB470267 [30]. The N-terminal and C-terminal regions of FliC are responsible for polymerization and secretion of flagella, while the central part is the antigenic part (surface-exposed) in the filament of flagella. Variation in the sequence of *fliC* may result in changes of flagella movement [29]. The sequence of *fliD* gene is highly conserved [50]. Here, there was no mutation in the sequences of *fliC* and *fliD* genes of the *C. difficile* isolate (Table 3).

In this study, the CE-ribotyping profile of this isolate did not correspond to any type of the previously known profiles in the ECDC-Leeds-Leiden reference *C. difficile* strain dataset (data not shown). However, toxinotyping revealed that this *C. difficile* belonged to toxinotype V which was also observed in 18.4% of the Iranian *C. difficile* isolates obtained from stool specimens of patients suffered from CDI [52]. One study was conducted in Iran in 2015 for detection of *C. difficile* in WWTPs. In that study, *C. difficile* was detected in different sites of wastewater treatment plants, but toxinotype and ribotype were not reported [2]. Norman et al.., (2011) also found toxinotype V in 84.5% of human wastewater *C. difficile* isolates in Texas, USA [13]. Another study from Slovenia showed 32 different ribotypes for *C. difficile* isolates from wastewater that belonged to toxinotypes 0, I, IX, V, and

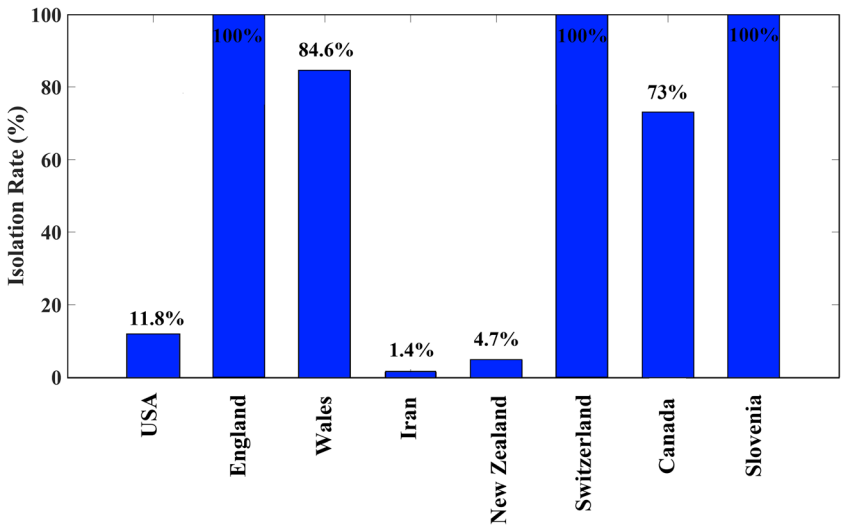

Fig. 3 The isolation rate (%) of *Clostridium difficile* from wastewater treatment plants (WWTPs) from different countries

XXIV [17]. Toxinotype V has not been considered as a major cause of CDI in hospitals; however, it was suggested that the rate of isolation of *C. difficile* toxinotype V from humans is increasing [13].

Conclusions

This study provided the evidence for presence of toxigenic *C. difficle* (toxinotype V) in the outlet of a WWTP in Iran. A combination of *slpA* sequence typing, *tcdC* genotyping and toxinotyping indicated that the *C. difficile* isolate may closely be related to RT078. Existence of a MDR and Tn*916*-like carrying-isolate of *C. difficile* in the outlet raises a question about the efficacy of wastewater treatment process. It should be noted that the discharge of outlet wastewater to environment can result in dissemination of *C. difficile* in settings other than hospitals and consequently may lead to CA-CDI in human populations and animals.

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Compliance with ethical standards The authors declare no conflict of interest.

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