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Molecular analysis and genotyping of pathogenicity locus in *Clostridioides difficile* strains isolated from patients in Tehran hospitals during the years 2007–2010

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ABSTRACT

Background & Aims: *Clostridioides difficile* (*C. difficile*) has been identified as the leading cause of antibiotic associated diarrhea (AAD). Co-carriage of an intact pathogenicity locus (PaLoc) with binary toxin genes in *C. difficile* strains seems to be linked with severe disease outcomes in the infected patients. Epidemiology of *C. difficile* infection (CDI) in hospital setting and knowledge about their genetic context help us to decrease *the morbidity, mortality*, and costs associated with *Clostridioides difficile infection.* In the present study was aimed to characterize genetic diversity of PaLoc among different *C. difficile* strains isolated from hospitalized patients and carriage of cytolethal distending toxin gene (*cdt*) in different hospitals.

Method: *C. difficile* strains were isolated from stool samples of inpatients referred to a reference laboratory from different hospitals and also outpatients with diarrhea, during 2008–2011. DNA was extracted from pure culture of the bacterium and PCR was performed for *tcdA*, *tcdB*, *tcdE*, *tcdC*, *tcdD*, and *cdu2* genes. Carriage of two binary toxin genes *cdtA*, *cdtB* was also determined in these strains. To find clonal strains, similarity of genotypes and integrity of PaLoc among the isolates was compared in each hospital.

Results: The intact PaLoc was found most frequently among the isolates in the outpatients (19/51, 37.2%, Group I), while incomplete PaLoc found mostly in patients who were hospitalized in the infectious diseases and internal diagnosis wards. *tcdA* and *tcdB* genes were detected in different combinations among the studied strains. These strains showed $tcdA^+B^+$, $tcdA^+B^-$, and $tcdA^-B^+$ genotypes in a frequency of 76.4% (39/51), 7.8% (4/51), and 17.6% (9/51), respectively. Analysis of gene composition of the PaLoc showed 19 distinct genotypes among the 51 strains. Accordingly, 38 strains were classified mainly into 6 regular groups, while the remaining strains showed heterogeneous patterns. $tcdC^-/tcdD^-$ constituted the most common genotypic group among the strains with partial PaLoc (7/51, 13.7%). A hypertoxigenic genotype, $tcdC^-/tcdA^+/tcdB^+$, was detected in 2 strains (2/51, 3.9%). The intact genotype was also detected in a *C. difficile* isolate from outpatients. *Cdt* encoding genes toxins was observed in low numbers of the strains (7/52, 13.5%). All of $cdtA^+B^+$ strains were belonged to PaLoc group 1 (intact genotype). Statistical analyses showed no correlation between particular genotypes and special wards of the hospitals (p value > 0.05).

Conclusion: Collectively, our results showed diversity of *C. difficile* strains in most wards of the studied hospitals.

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Diversity of PaLoc genotypes in the strains that isolated from the same wards proposed endogenous routes of the infection, as common cause of CDI in these patients.

1. Introduction

Clostridioides difficile is the etiologic agent of pseudomembranous colitis (PMC) and the most common cause of nosocomial antibiotic associated diarrhea (AAD) (Spigaglia and Mastrantonio, 2002). Typically, the disease develops in elderly patients with current or previous hospitalization and recent antibiotic therapy (Rupnik, 2008). Two structurally similar toxins, denoted as TcdA (308 kDa) and TcdB (270 kDa), are the main virulence determinants associated with *Clostridioides difficile*-associated disease (CDAD). Most pathogenic strains of *C. difficile* produce both toxins (A^+B^+) (Drudy et al., 2007). These two virulence factors belong to a large Clostridial cytotoxin family known as glucosylating *toxins, which its main effect is disruption of* actin cytoskeleton (Spigaglia and Mastrantonio, 2002).

tcdA and *tcdB* (genes that encode TcdA and TcdB toxins) are part of a 19.6-kb genetic locus that encoded along with three other genes in the pathogenicity locus (PaLoc), *tcdD*, *tcdE*, and *tcdC* and the open reading frames (ORFs) for the insertion sequences, *cdu-2*, *cdu-2′*, *cdd-2, cdd-3,* and *cdd-4* (Spigaglia and Mastrantonio, 2002). In nontoxigenic strains, the pathogenicity locus (PaLoc) is not present, and a 115-bp DNA fragment is found between the 2 insertion sequences. The ORFs for the insertion sequences, *cdu*-2/2′ and *cdd*-2-3, located orderly upstream and downstream of the pathogenicity locus (PaLoc), are present in nontoxigenic strains (Cohen et al., 2000; Spigaglia and Mastrantonio, 2002). *tcdD* is a positive regulator of toxin expression (Shiloh et al., 1997). This gene is found upstream of *tcdB* and is coordinately expressed with *tcdB* and *tcdA* (PMCID: PMC1082799). *tcdC* is an anti-sigma factor that lies downstream of *tcdA* and is highly expressed in early exponential phase, but declines as growth moves into the stationary phase. It is documented that TcdC has function as a negative regulator in toxin expression (PMID: 17542920, PMID: 22522680). TcdE is a holin family protein that may be involved in the export of TcdA and TcdB from the cell. However, there are still controversial issues in relation to the role of TcdE (Smits, 2013).

Different studies described variations in *C. difficile* strains for carriage of toxin A and B or their positive and negative regulator genes. Despite the fact that *C. difficile* strains with diversity in PaLoc can still be associated with clinical diseases, a few epidemiological data on their circulation are reported and little is known about PaLoc accessory gene variants. In this study, we analyzed several strains of *C. difficile* to determine their diversity in the pathogenicity locus, identify the variants, and determine possible correlations between these strains in the studied hospitals.

2. Materials and methods

A total of 51 strains of *C. difficile* from 718 stool samples were included in this study. All the strains were isolated from patients with diarrhea who referred to reference laboratory of *C. difficile* in Ayatollah Taleghani Hospital in Tehran, Iran during 2007–2010. All the patients informed about study on their samples and signed a standards consent form that were approved by Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

The strains were cultured on Cycloserine Cefoxitin Fructose Agar (CCFA), then incubated at 37 °C for 48 h under anaerobic condition in an anaerobic jar with gas pack type A (Merck, Germany) or Anoxomate system (Mart, Netherland). To confirm identity of *C. difficile* strains, pure cultures containing 1–3 mm diameter rounded edges colonies that showed white to yellow color were subjected to DNA extraction by boiling method (Rupnik et al., 2001). Briefly, a loop full colony of each strain of *C. difficile* isolates were suspended in 500 µl of distilled water and vortexed. Supernatant was thrown away followed by centrifugation for 10 min at 13000*g* at 4 °C. The pellets were mixed with 100 µl of distilled water, and centrifuged at 13000*g* for 8–10 min after boiling in water bath for 10 min. The supernatant containing bacterial DNA was transferred to a new sterile tube and stored at −20 °C until further use.

2.1. Detection of PaLoc gene cluster by polymerase chain reaction

To confirm entity of the strains and their diversity in PaLoc, *cdd3* universal primers and those specific for *tcdA*, *tcdB*, *cdtA*, *cdtB*, *tcdE*, *tcdC*, *tcdD*, and *cdu2* genes were used for amplification by polymerase chain reaction. The list of primers sequences and length of their products are shown in Table 1. Amplification of *cdd3* and PaLoc gene clusters was performed as follows. In the case of *cdd3*, *tcdA*, and *tcdB*, conventional *singleplex PCR* was used. The reaction mixtures contained 2.5 µl 10× PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 0.5 µl of dNTP mix (25 mM), defined amounts of forward and reverse primers (20 pM, 0.5 µl), 0.5 µl of Taq DNA Polymerase (5 unit/µl), 1 µl of template DNA

Table 1
Nucleotide sequences of the primers used for identification of *C. difficile* strains and their diversity in the PaLoc.

Primers name	Primer	Sequencing (5′ to 3′)	Product size (bp)	Reference
cdd3	Tim6	F-TCCAATATAATAAATTAGCATTCC	622	(Spigaglia and Mastrantonio, 2002)
	Strupp6	R-GGCTATTACACGTAATCCAGATA		
TcdA	TA1	F-ATGATAAGGCAACTTCAGTGG	624	
	TA2	R-TAAGTTCCTCCTGCTCCATCAA		
TcdB	TB1	F-GACCTGCTTCAATTGGAGAGA	412	
	TB2	R-GTAACCTACTTTCATAACACCAG		
CdtA	CdtA-f	F-AGGATTATTTACTGGACCATTTG	399	
	CdtA-r	R-AACGGATCTCTTGCTTCAGTC		
CdtB	CdtB-f	F-CTTAATGCAAGTAAATACTGAG	501	
	CdtB-r	R-AACGGATCTCTTGCTTCAGTC		
tcdE	Tim1	F-CTTTAAGTGCAATAAAAAGTCGTA	262	
	Strupp1	R-GGTAATCCACATAAGCACATATT		
tcdC	Tim2	F-GCACCTCATCACCATCTTCAA	345	
	Strupp2	R-TGAAGACCATGAGGAGGTCAT		
tcdD	Tim3	F-AAAAGCGATGCTATTATAGTCAAA	300	
	Strupp3	R-CCTTATTAACAGCTTGTCTAGAT		
Cdu2	Tim5	F-CCACAGATGCTTTTAGCAGCAA	162	
	Strupp5	R-TCCATCACTGCTCCAGCTAT		

and 16 µl doubly deionized water was prepared. PCR conditions were included one cycle of initial denaturation (95 °C, for 8 min), followed by 35 cycles of denaturation (95 °C, 1 min), annealing (58 °C, 40 s), extension (72 °C, 1.5 and 1.2 min for *cdd3* and *tcdA/B*, respectively), and one cycle of final extension (72 °C, 10 and 5 min for *cdd3* and *tcdA/B*, respectively). Multiplex PCR reaction was used for detection of PaLoc gene cluster. A mixture of 10 µl 10× PCR buffer, 3 µl of $MgCl_2$ (50 mM), 1 µl of dNTP mix (25 mM), defined amounts of forward and reverse primers (20 pM, 1 µl for Tim5/*cdu2*, 1.3 µl for Tim3/*tcdD*, 1.3 µl for Tim1/*tcdE*, and 0.7 µl for Tim2/*tcdC*, 0.5 µl of Taq DNA polymerase (5 unit/µl), and 2 µl of template DNA was prepared. The amplification was done as follows: 5 min initial denaturation, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C, extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 4 min. PCR conditions for *cdt*A was included a denaturation at 95 °C for 1 min, annealing at 58.5 °C, extension at 72 °C for 45 s, and one cycle of final extension at 72 °C for 10 min. PCR conditions for *cdt*B was included a denaturation at 95 °C for 1 min, annealing at 58.9 °C, extension at 72 °C for 35 s, and one cycle of final extension at 72 °C for 10 min. Amplified products of the singleplex and multiplex PCR were orderly visualized in 1% and 2% agarose gels in Tris base boric acid EDTA (TBE) after staining in an ethidium bromide solution (10 µg/ml) for 30 min under Gel documentation (Bio-Rad). In all cases, a negative control containing all PCR reagents (without DNA) was used to monitor the contamination. *C. difficile* strain VPI 10463 was used as a positive control for *cdd3*, *tcdA* and *tcdB* genes.

2.2. Sequencing of PaLoc gene cluster

To verify correct amplification of *tcdE*, *tcdC*, *tcdD*, and *Cdu2* genes, a few of the amplicons were sequenced. Obtained sequences were analyzed by using MEGA4 software and compared with NCBI databank. All the sequences were submitted to the GenBank nucleotide database for the purpose of registration and receiving accession numbers. Edraw max software was used to draw genetic maps related to PaLoc of the strains.

2.3. Statistical analysis

To analyze association between the PaLoc genotypes and *C. difficile* transmission among and within the hospitals/wards, statistical analysis was performed using SPSS 17.0 software. p value $< .05$ was considered statistically significant.

3. Results

Out of the studied *C. difficile* strains, the intact PaLoc, presenting $Cdu2^+/tcdD^+/tcdB^+/tcdE^+/tcdA^+/tcdC^+/cdd3^+$ genotype, was found most frequently among the isolates in the outpatients, while incomplete PaLoc found mostly in patients who were hospitalized in the infectious diseases and internal diagnosis wards (Table 2). *tcdA* and *tcdB* genes were detected in different combinations among the studied strains. These strains showed $tcdA^+B^+$, $tcdA^+B^-$, and $tcdA^-B^+$ genotypes in a frequency of 76.4% (39/51), 7.8% (4/51), and 17.6% (9/51), respectively.

Accurate amplification of the genes targeted was verified by sequencing. The following GenBank accession numbers were obtained accordingly (KM047900, KM047901). Analysis of nucleotide sequences showed a variant of *tcdC* with an insertion in nucleotide positions 372–391. Analysis of gene composition of the PaLoc showed 19 distinct genotypes among the 51 strains. Accordingly, 38 strains were classified mainly into 6 regular groups, while the remaining 13 strains designated as “heterogeneous group” showed different patterns (Table 2, Fig. 1). One of the strains in heterogeneous group was *cdu2* negative. $tcdC^-/$

Table 2
Distribution of PaLoc (Groups 1–6) among the *C. difficile* isolates from patients in different hospital wards.

Wards	Frequency	Percent	Intact PaLoc	Partial PaLoc	PaLoc groups[a.]	*cdtA* and *cdtB*
			n/N, %	n/N, %		N, %
Internal diagnosis unit	9	17.6	5/9, 55.5%	4/9, 44.4%	Hospital 1: Group 1 (1) Hospital 5: Group 6 (2) Hospital 6: Group 1 (1) Hospital 9: Group 1 (1) Hospital 12: Group 1 (1) Hospital 15: Group 2 (2) Hospital 21: Group 1 (1)	$cdtB^+$ (1)
Infectious Diseases	4	7.8	3/4, 75%	1/4, 25%	Hospital 4: Group 3 (1); Group 1 (1) Hospital 9: Group 1 (1) Hospital 13: Group 1 (1)	$cdtB^+$ (1) $cdtA^+/cdtB^+$ (1) $cdtA^+$ (1)
Surgery	1	2	1/1, 100%	0/1, 0%	Hospital 6: Group 3 (1)	$cdtB^+$ (1)
Intensive care unit	9	17.6	7/9, 77.7%	2/9, 22.2%	Hospital 2: Group 1 (1) Hospital 6: Group 1 (1) Hospital 7: Group 1 (1) Hospital 8: Group 1 (1) Hospital 9: Group 1 (1) Hospital 10: Group 1 (1) Hospital 14: Group 1 (1) Hospital 20: Group 3 (2)	$cdtA^+/cdtB^+$ (1) $cdtA^+$ (1)
Pediatric	1	2	1/1, 100%	0/1, 0%	Hospital 9: Group 1 (1)	
Oncology	3	5.8	3/3, 100%	0/3, 0%	Hospital 9: Group 5 (1) Hospital 20: Group 5 (1) Hospital 19: Group 5 (1)	
Cardiac intensive care Unit	1	2	1/1, 100%	0/1, 0%	Hospital 3: Group 1 (1)	
Gynecology	3	5.8	0/3, 0%	3/3, 100%	Hospital 17: Group 2 (3)	
Gastroenterology	4	7.8	4/4, 100%	0/4, 0%	Hospital 4: Group 4 (1) Hospital 9: Group 4 (1) Hospital 11: Group 1 (1) Hospital 21: Group 4 (1)	
Outpatient clinics	1	2	1/1, 100%	0/1, 0%	Hospital 9: Group 1 (1)	
Orthopedics	2	3.9	0/1, 0%	2/2, 100%	Hospital 16: Group 2 (2)	
Total	38/51	74.5	26/38, 68.4%	12/38, 31.6%		7

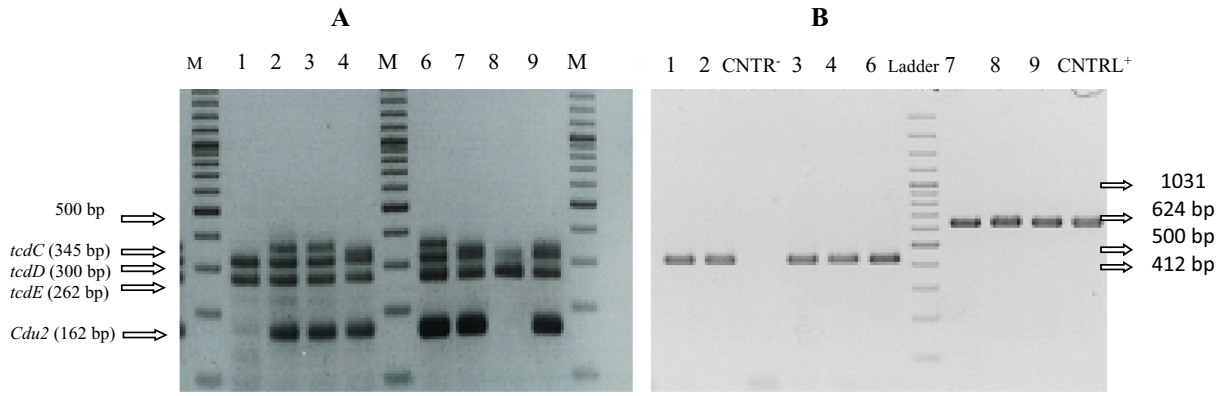

Fig. 1. PCR for analysis of PaLoc associated genes *cdu2, tcdD, tcdE, tcdC, tcdA* and *tcdB* among different *C. difficile* strains.
DNA extracts from pure cultures of *C. difficile* strains were used for detection of PaLoc genes in *C. difficile* strains. Panel A: Multiplex-PCR for *cdu2* (162 bp), *tcdE* (262 bp), *tcdD* (300 bp) and *tcdC* (345 bp); Panel B: PCR product of *tcdA* (624 bp) and *tcdB* (412 bp). CTRL$^+$, Positive control; CTRL$^-$, negative control; DNA molecular weight marker: Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder.

tcdD$^-$ (Group 2) constituted the most common genotypic group among the strains with partial PaLoc (7/51, 13.7%). A hypertoxigenic genotype, *tcdC*$^-$/*tcdA*$^+$/*tcdB*$^+$, was detected in 2 strains (2/51, 3.9%). The intact PaLoc was mostly detected in the Infectious Diseases (7.8%), ICU (17.6%), Gastroenterology (7.8%), and Internal diagnosis units (17.6%) in the studied hospitals (Table 2). *C. difficile* isolates from outpatients also showed intact genotype (2%). Statistical analyses showed no correlation between particular genotypes and special wards of the hospitals (p value > .05). Existence of *cdt* genes were observed in low numbers of the strains (7/52, 13.5%). All of *cdtA*$^+$*B*$^+$ strains were belonged to PaLoc group 1 (intact genotype) (Table 2, Fig. 2).

Statistical analysis showed lack of association between the characterized PaLoc genotypes in *C. difficile* strains among the studied wards and occurrence of diarrhea in the studied patients (Fig. 3). Genotype I was detected as more common genotype in the Infectious diseases and Internal diagnosis units.

4. Discussion

The *C. difficile* PaLoc shows a mosaic structure in different strains. This diversity occurs during homologous recombination, gene transfer, and genomic evolution (Knight et al., 2015).While this bacterium is distributed worldwide equally, reports of outbreaks by hypertoxigenic strains that showed mutations in PaLoc and their link with lethal infections have attracted the scientists and clinicians' attention in recent year (Paquette et al., 2015). Although the presence of PaLoc in strains of *C. difficile* is an indicator of their pathogenicity (Kuijper et al., 2006), different clinical outcomes are observed through infection with varying strains. In our study, diversity in PaLoc was detected among the clinical isolates from the studied hospitals. This divergence, either among the strains from the hospitals or within the wards, proposed acquisition of CDI through an endogenous source of the strains in most of these patients, which was mediated by usage of broad spectrum antibiotics. Similar to our results, Widmer A.F. et al. showed a low rate of transmission of toxigenic *C. difficile* in a tertiary academic care center (Widmer et al., 2016). In other studies contact with symptomatic patients and hospital environment was shown as most common routs of *C. difficile* transmission in hospital settings (Zhou et al., 2014).While our results showed similarity of the strains in some wards of the studied hospitals, phylogenetic analysis using approved typing methods are needed to provide data regarding dissemination routes of the strains. This similarity was related to Infectious Diseases and Internal diagnosis units in different hospitals, which was mainly linked to the PaLoc group 1 strains.

Despite the presence of regulatory gene *cdtR* among most strains of *C. difficile*, many of them contain only truncated forms of *cdtA* and *cdtB* (Gupta et al., 2013). Variation of Cdt-locus was shown among the *C. difficile* strains in our study, in that *cdtA*$^-$*B*$^-$ genotype was more frequent in the studied strains. This diversity was also observed among different strains in each ward (*cdtA*$^+$, *cdtB*$^+$, *cdtA*$^+$*B*$^+$, and *cdtA*$^-$*B*$^-$). Although mortality of CDT-positive strains of *C. difficile* with intact PaLoc was reported higher compared with CDT-negative ones in patients with CDI (Bacci et al., 2011), CDT-encoding strains with partial PaLoc (either *tcdA*$^-$*B*$^+$ or *tcdA*$^-$*B*$^-$) were also reported from cases of CDI (Arumugam et al., 2011). The existence of intact PaLoc in most of the strains that carried binary toxin genes highlighted a public health concern regarding the spread of these strains in our hospitals and the community, which is clinically important. This can increase risk of severe diseases in our hospitals. The prevalence of Cdt-encoding strains was 13.5% among the strains studied, which was relatively lower than those reported from European countries (17.2%–23%) (Barbut et al., 2007;. Bauer et al., 2011; Gerding et al., 2014; Keel et al., 2007). *Clostridioides difficile* infection in Europe: a hospital-based survey (Smits, 2013), but similar to those reported recently from Australia (12.5%) (McGovern et al., 2017). This frequency was higher than the last report from Iran, in which CDI by *tcd*A$^+$/B$^+$/*cdt*$^+$ strains was

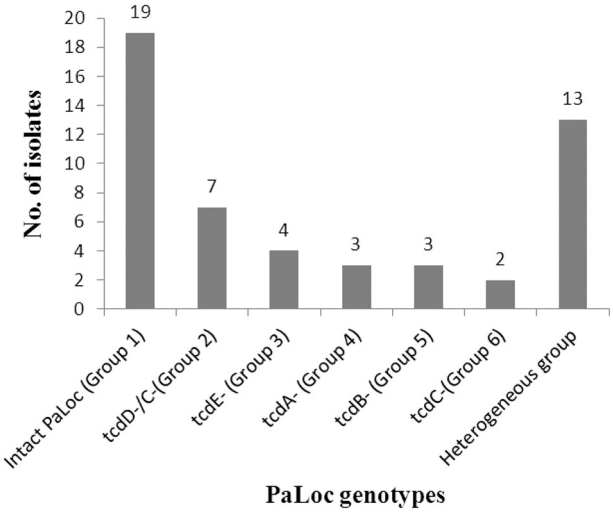

Fig. 2. Diversity of genes in PaLoc of *C. difficile* strains.
The figure shows gene arrangement of the PaLoc in 51 clinical strains of *C. difficile* strains. Nineteen distinct genotypes obtained, among them thirty eight strains were classified mainly into 6 regular genotypic groups designated as groups 1–6. Thirteen strains with different patterns placed in a group designated as "Heterogeneous group".

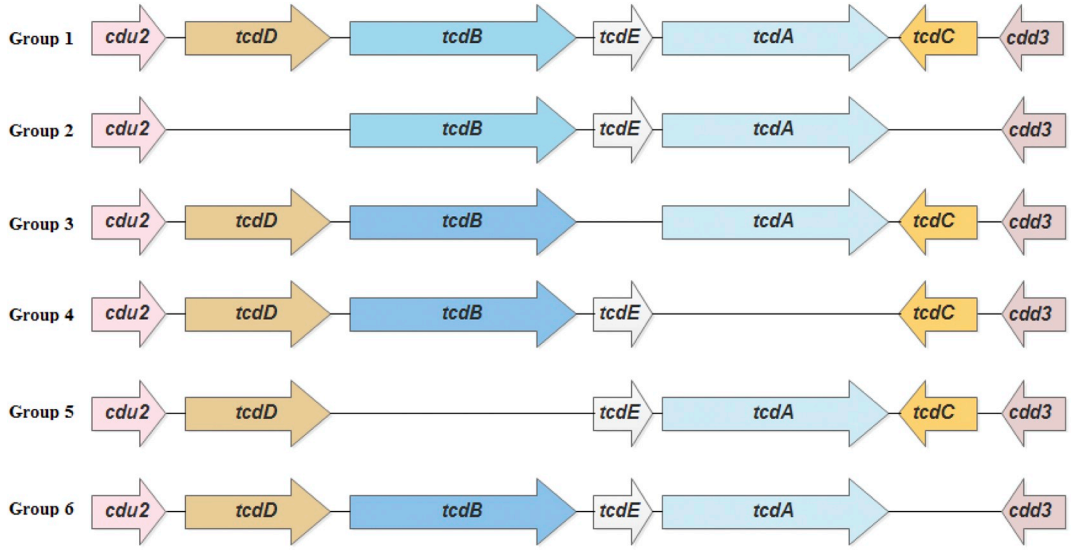

Fig. 3. Genetic organization of PaLoc in *Clostridioides difficile* strains obtained from symptomatic patients in different hospital wards.
Group 1, *Cdu2*$^{+}$/*tcdD*$^{+}$/*tcdB*$^{+}$/*tcdE*$^{+}$/*tcdA*$^{+}$/*tcdC*$^{+}$/*cdd3*$^{+}$; **Group 2**, *Cdu2*$^{+}$/***tcdD***$^{-}$/*tcdB*$^{+}$/*tcdE*$^{+}$/*tcdA*$^{+}$/***tcdC***$^{-}$/*cdd3*$^{+}$; **Group 3**, Cdu2^{+}/tcdD^{+}/tcdB^{+}/**tcdE**$^{-}$/tcdA^{+}/tcdC^{+}/cdd3^{+}; **Group 4**, Cdu2^{+}/tcdD^{+}/tcdB^{+}/tcdE^{+}/**tcdA**$^{-}$/tcdC^{+}/cdd3^{+}; **Group 5**, Cdu2^{+}/tcdD^{+}/**tcdB**$^{-}$/tcdE^{+}/tcdA^{+}/tcdC^{+}/cdd3^{+}; **Group 6**, Cdu2^{+}/tcdD^{+}/tcdB^{+}/tcdE^{+}/tcdA^{+}/**tcdC**$^{-}$/cdd3^{+}.

shown in 2% of the patients (Azimirad et al., 2018; Zarandi et al., 2017).Comparative analysis of defined genotypes of *C. difficile* revealed that, with the exception of group 4 ($A^{-}B^{+}$) and 5 ($A^{+}B^{-}$), most of these groups contain toxinogenic variants ($A^{+}B^{+}$). Group I (Intact PaLoc) was constituted the more frequent genotype among the toxinogenic strains. The number of genotypic groups that were identified in each ward varied widely, which might be consistent with evolutionary events that occurred independent of PaLoc acquisition followed by clonal expansion or endogenous source of the infection. Absence of *tcdC* in *tcdA*$^{+}B^{+}$ strains, which could be consistent with hypervirulent variants of *C. difficile*, was detected in a lowest frequency (3.9%). A higher rate of frequency of *tcdC* deletion (30%) was reported in a study (Sester et al., 2010). This inconsistency could be explained by the method used for detection of deletion in 18-bp and other nucleotide positions of *tcdC* and general PCR for detection of *TcdC*. Although association between deletions in *tcdC* and hyperproduction of cytotoxins was rejected recently by some studies, detection of this genotype in an ICU in the studied hospital showed a risk for severe disease. While our results don't show a relationship between the presence and absence of genetic loci in these strains and large-scale deletions, analysis of the PaLoc at sequence level can reveal evolutionary events of this chromosomal area. Detection of sources and origins of the CDI, either in animals, the environment, or humans, will help us to control dissemination of pathogenic variants.

4.1. Conclusion

Collectively, our results showed diversity of *C. difficile* strains in most wards of the studied hospitals. Diversity of PaLoc genotypes in the strains that isolated from the same wards proposed endogenous routes of the infection, as common cause of CDI in these patients. Further studies at sequence level are needed to establish this finding. Infection with Cdt-encoding strains representing intact PaLoc and *tcdC* negative variants, which showed higher link with mortality, highlighted designing new strategy for prevention of their spread.

Conflict of interest

All authors declare there is no conflict of interest.

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