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Characteristics of *Clostridioides difficile* colonization in Ilamian children

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Key words: Clostridioides difficile, bacterial colonization, children
Parole chiave: Closteridioides difficile, colonizzazione batterica, bambini

Abstract

Background. *The increasing colonization with Clostridioides difficile in paediatric hospitalized population is a well known event; however, its prevalence in Iranian children has not effectively been identified yet.*
Objective. *The objective of this study was to determine the intestinal-carriage rates of C. difficile and molecular characterization of C. difficile in the Ilamian pediatric population from May 22, 2018, until September 22, 2018.*
Materials and Methods. *Eighty samples were obtained from 40 children aged <5 years, at day 0 of their hospitalization (N=40 samples), to determine community-associated colonization, and then at day 5 days after hospitalization (N=40 samples), to determine healthcare associated colonization. The stool samples were examined for C. difficile, and isolated strains were evaluated for production of Clostridial toxins A/B and molecular characterizations.*
Results. *The colonization rates of C. difficile and toxigenic C. difficile were 10% (8/80) and 3.75% (3/80), respectively. Based on the age group, the intestinal-carriage rates of C. difficile were 37.5, 50, and 12.5% in children ≤ 1, 1-3, and 3-5 years old, respectively. Our findings have revealed eight distinct ribotypes. Our findings have revealed eight distinct ribotypes of C. difficile isolates. Three out of 8 (37.5%) of C. difficile isolates were considered as community-associated colonization and belonged to ribotypes 7, 8, and 9.*
Conclusion. *Our findings suggest the need of confirmation by further epidemiological studies in Iranian children. Given that the 37.5% of cases observed were community-associated, estimates of the incidence of C. difficile infections, that include only hospitalized children, may largely underestimate the burden of disease in children.*

Introduction

Clostridioides (previously *Clostridium*) *difficile,* as a gram-positive, spore-forming, cytotoxin-producing anaerobic bacillus was first discovered from the stool of healthy neonates in 1935 by Hall and O'Toole (1). The pathogenic locus (PathLoc) of *C. difficile* is an area containing three accessory genes that are able to encode the factors regulating the production of toxins. Changes in Pathoc's structure produce several toxinotypes (2-5). Toxin A (*tcdA*, enterotoxin, 308 kDa) and toxin B (*tcdB*, cytotoxin, 270 kDa) are the

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major virulence factors of *C. difficile* (2-5). In addition, some *C. difficile* strains are able to produce binary toxins (CDT). CDT is made up of two different subunits: an enzyme subunit called CDTa and a binding subunit called CDTb. It is synthesized by two different genes, cdtA and cdtB, which are located outside the PathLoc (2, 6). The pathogenic role of *C. difficile* in children remains poorly characterized, and cannot be overlooked. It is important to know the risk factors related to the infection, the correct diagnostic-therapeutic approach and therapeutic perspectives against the pathology of growing interest for the pediatric population.

There has been a dramatic change in the epidemiology of *C. difficile* infection (CDI) in children since the 1970s. This includes: a higher incidence of CDI in hospitalized children, the emergence of community-acquired infections, and a more severe degree of the infection with the NAP1 strain (7).

A higher rates of colonization with toxigenic *C. difficile* strains in neonates and young children have been determined; however, they rarely begin with any symptoms. The immaturity of the toxin receptor sites may also play a role in the lack of a typical disease in neonates (8).

However, genomic analyses have determined that the newborn's gut is colonized with maternal and placental microbiome at the time of birth rather than being sterile (9).

It has been demonstrated that 60-70% of infants are colonized with *C. difficile* (10, 11).

The colonization rate of *C. difficile* among healthy infants aged < 24 months has shown a peak of 48.5% (12). In previous reports (13-15), infants aged < 1 year showed peak rates of *C. difficile* colonization (14-44%), while these rates decreased to 3.5-10% at the age of one year.

Additionally, asymptomatic carriers of *C. difficile* are able to spread the infection to other children (10, 11). The annual incidence of *C. difficile* infection has generally increased among hospitalized children (16). The spread of *C. difficile* within healthcare centers occurs via contamination of healthcare workers' hands after contact with the skin of patients or with contaminated environmental surfaces including beds, commodes, bathtubs, telephones, computers, light switches, sinks, tables, and window sills (17, 18). Despite the commensal nature of *C. difficile*, it is significantly associated with diarrhoea, pseudomembranous colitis and reactive arthritis in paediatric outpatients or inpatients (3, 13). Thus, it is urgent to control the spread of this infection in healthcare centers. In general, due to the inadequate laboratory diagnostic capacity in Iran, and a limited familiarity with *C. difficile,* colonization has not been well known in the pediatric population. With this background, in this study, we sought to highlight the intestinal-carriage rates of *C. difficile* and molecular characterization of *C. difficile* in Ilamian pediatric population at days 0-5 after admission.

Methods

Specimen collection and study design

In the prospective of a cross-sectional study, a total of 80 unformed stool specimens were collected from 40 hospitalized pediatric patients at day 0 (40 stools specimens) and within 5 days after admission (40 stools specimens) at pediatrics ward of Imam Khomeini hospital in Ilam, Iran from May 22, 2018 until September 22, 2018. Inclusion criteria were children less than 5 years old and apparently without underlying diseases.

The parents of children completed a "verbal" and "written" questionnaire containing different clinical and personal data such as clinical symptoms, use of antibiotics and underlying conditions. This project was approved by the Ilam University Human Ethics committee.

Isolation and identification of isolates

Stool specimens were transported to the laboratory and processed immediately. They were directly cultured on CCFA agar Plate (CCFA: cycloserine-cefoxitin-fructose agar, Conda, Spain) supplemented with 7% defibrinated sheep blood and selective components (8 mg/L cefoxitin and 250 mg/L cycloserine), followed by alcohol shock (~1 g or 1 mL of stool was added to an equal volume of absolute ethanol) for 1 h at room temperature (18). The plates were incubated anaerobically using Gas-Pac anaerobic jars (Merck, Germany) at 37°C for 48 h. The suspected isolates were considered as *C. difficile*, based on characteristic phenotype (circular yellow or grey-white colonies with raised centers with irregular filamentous or opaque edges, Gram and spore staining, and typical odour: horse barn) (18). Stock cultures were kept in Brain heart infusion (BHI) broth (Conda, Spain) containing 20% glycerol at -80 °C for further analysis.

Primer design

In this study the primers were designed using the Genscript software (http://genscript.com/ssl-bin/app/primer). They were used for designing the 16S rDNA (as housekeeping gene), toxin A (*tcdA*), and toxin B (*tcdB*) genes (Table 1). A basic local alignment search tool (BLAST) was performed on these primers in order to evaluate sequences and test specificity of the primers. The primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

DNA extraction

Total genomic DNA of *C. difficile* isolates was extracted using boiling method as previously described (19). DNA purity, quality and quantity were measured using NanoDrop (Eppendorf, Germany). Whole extracted DNAs were immediately stored at -20 °C.

Molecular detection of C. difficile isolates

The *C. difficile* isolates were evaluated by PCR for detection of 16S rDNA (as housekeeping gene), toxin A (*tcdA*), and toxin B (*tcdB*) genes. Additionally, PCR amplification was performed using specific primers for *cdtA* and *cdtB* genes as previously described by Stubbs et al. (20). PCR amplification reaction and the PCR protocols for detection of 16S rDNA *tcdA*, and *tcdB* genes were listed in the Supplementary data 1, 2 and 3. PCR reactions were run on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA). Then PCR products were analyzed by Gel document Bio-Rad's Gel Doc XR+ system. All gels were run under standard conditions on 1.5% agarose and stained with EcoDye™ DNA Staining Solution (BIOFACT, South Korea). The PCR purified products were subjected to DNA sequencing by Bioneer (Bioneer, South Korea). The obtained sequences were then analyzed by Chromas 2.5 software (Technelysium, Tewantin, Australia; http://technelysium.com.au/wp/chromaspro/). Finally, the sequences were evaluated using the Blastn algorithm at the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed/). *C.*

Table 1 - The sequences of primers used in the study

Primer	Sequence (5′→3')	Product Size (bp)	Ref.
tcdA	F: CCAACACCTTAACCCAGCCA	165	This study
	R: ATTGTGGAGCGAGCTTCTGG		
tcdB	F: AGGTGCAGCAATCAAAGAGC	409	
	R: ACCTGAGCCACCTTCCATTC		
16srRNA	F: GAATGAGCTGACCCCCAACA	465	
	R: GCTCAGTCAAGGCCTCCTTT		

Supplementary data 1 - PCR conditions used for *amplification* of the 16S rDNA gene

Reagent	Volume(µL)	Final concentration
PCR Master mix(2X)	13	5.2X
Primer F (10 pM)	1	0.4 pM
Primer R (10 pM)	1	0.4 pM
Deionized water	7	-
DNA Template (100 ng/µL)	3	20 ng/µl
Total	25	-

Step	Temperature (‘c)	Time (min)	Number of cycles
Initial Denaturation	95	5m	1
Denaturation	95	45s	39
Annealing	58.5	50s	
Extension	72	45s	
Final extension	72	10m	1

Supplementary data 2 - PCR conditions used for *amplification* of the *tcdA* gene

Reagent	Volume(µL)	Final concentration
PCR Master mix(2X)	13	5.2X
Primer F (10 pM)	1	0.4 pM
Primer R (10 pM)	1	0.4 pM
Deionized water	7	-
DNA Template (100 ng/µL)	3	20 ng/µl
Total	25	-

Step	Temperature(‘c)	Time (min)	Number of cycles
Initial Denaturation	95	5m	1
Denaturation	95	45s	3
Annealing	59.5	45s	
Extension	72	40s	
Final extension	72	10m	1

difficile ATCC 9689 were used as a positive control strain.

Molecular characteristics of C. difficile isolates

Primers 16S (5’-GTGCGGCTGGAT CACCTCCT-3’) and 23S (5’-CCCTGCAC-CCTTAATAACTTGACC-3’) were used in agarose gel-based PCR-ribotyping and performed as described by Bidet *et al.* (1999) (21). In brief, the amplification reaction was performed in a volume of 25 µL containing 12.5 µL HotStarTaq Mastermix 2X (Solis BioDyne, Estonia), 8.5 µL sterile distilled water, 1 µL of each primer (5µm/µL), and 2 µL of the template DNA (50 ng/µl). Amplification was carried out in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) of 24 cycles each including 95 ° C, 15 min for initial denaturation, 95 °C,1 min for denaturation, 57 °C, 1 min for annealing, 72 °C, 1 min for extension step, plus a 72 °C ,30 min for final extension step. The lanes were aligned and analyzed with the computer software Gel compare version 4.0 (Applied Maths, Kortrijk, Belgium).

Supplementary data 3 - PCR conditions used for *amplification* of the *tcdB* gene

Reagent	Volume(µL)	Final concentration
PCR Master mix(2X)	13	5.2X
Primer F (10 pM)	1	0.4 pM
Primer R (10 pM)	1	0.4 pM
Deionized water	7	-
DNA Template (100 ng/µL)	3	20 ng/µl
Total	25	-

Step	Temperature(‘c)	Time (min-s)	Number of cycles
Initial Denaturation	95	m5	1
Denaturation	95	50s	37
Annealing	58	50s	
Extension	72	50s	
Final extension	72	10m	1

Results

Colonization rate of C. difficile

The colonization rate of *C. difficile* in children was 20% (8/40) according to the phenotypic and PCR-based sequencing (16S rDNA). Using PCR assay, 3/8 *C. difficile* isolates were toxigenic (*tcdB* positive/*tcdA* negative). None of them possessed the genes encoding the binary toxin.

Three isolates obtained from three patients were *C. difficile* positives (non-toxigenic) among samples collected at day 0 after admission (community acquired) and 5 isolates obtained from 5 patients tested positive after 5 days of admission (healthcare associated acquisition). All of the eight *C. difficile* were obtained from different patients.

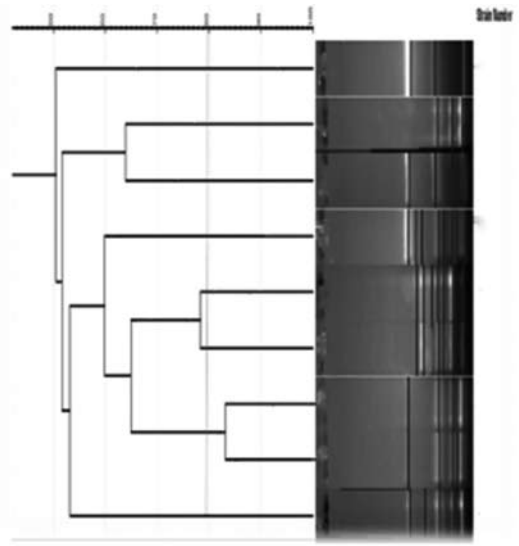
The demographic characteristics in children are shown in Table 2. The intestinal-carriage rates of *C. difficile* in 40 children ≤ 1, 1-3, and 3-5 years old were estimated 7.5, 10, and 2.5%, respectively.

Molecular typing of toxigenic C. difficile

All of *C. difficile* strains belonged to different PCR ribotype patterns (Figure 1). We have disclosed 8 unique ribotypes (RTs) of *C. difficile* dispersed. Band patterns of PCR ribotype amplicon were observed by agarose gel electrophoresis in Supplementary data 4. There was no identity between the strains and no dominant ribotype was identified. The highest homology (83%) was observed between R2 and R3. Three of isolates were determined as community-associated colonization and belonged to RTs 7, 8, and 9.

Discussion

Over the past two decades, the epidemiology of *C. difficile* infection in children underwent a chang (7, 11, 13). The detailed role of *C. difficile* in children remains less than well-defined. Generally, CDI in children is described as colonization without disease during infancy, and rarely a symptomatic infection (8, 22). CDI in children is associated with increased mortality, length of stay and hospital costs (8, 22). Mostly, antibiotic-associated diarrhoea caused by *C. difficile* is strongly linked to children, and symptoms are more likely to be severe in the presence of co-morbidities such as immunosuppression, haematological



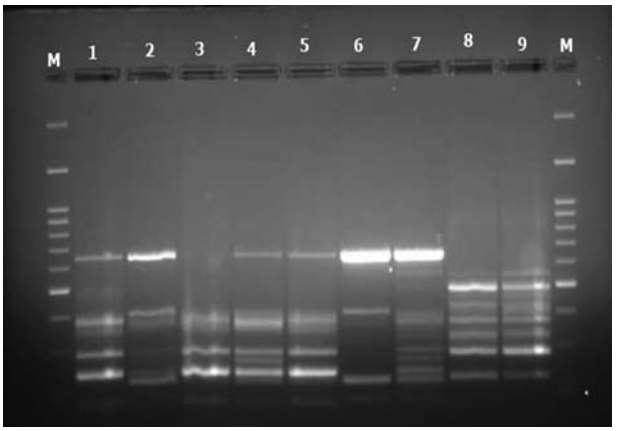
Ribotype	Toxin Profile	ID
R9	A-B-	9
R8	A-B-	8
R7	A-B-	7
R6	A-B-	6
R5	A-B+	5
R4	A-B-	4
R3	A-B+	3
R2	A-B+	2
R1	A+B+	positive control

Figure 1 - Dendogram of PCR ribotypes produced using GelCompar Software (Applied Maths, Belgium).

Table 2 - Demographic and clinical characteristics of 40 pediatric patients

Characteristic	Number of patients	N. of toxigenic isolates	N. of non-toxigenic isolates
Sex			
female	14	1	2
male	26	2	3
Age (yrs)			
≤ 1	14	1	2
1-3	20	2	2
3-5	6	0	1
Appearance of stools			
liquid	18	2	3
Mucoid	9	1	1
Bloody	3	0	1
Formed	10	0	0
Hospital ward			
Gastroenterology	5	1	0
Infectious ward	13	1	1
Internal medicine	5	0	2
Other	17	1	2
Exposure to antibiotics			
Clindamycin	6	1	1
amoxicillin	5	0	1
ceftriaxone	5	1	0
aminoglycosides	4	0	0
ampicillin	8	1	2
metronidazole	4	0	0
cefotaxime	1	0	0
co-trimoxazole	3	0	1
Other	4	0	0

Supplementary data 4 - PCR ribotype amplicons banding patterns as observed by agarose gel electrophoresis



malignancies and bowel disorders (23-26). It is assumed that asymptomatic *C. difficile* colonization in children is a significant reservoir for spread of the infection to adults (8, 12, 26).

The carriage rate and age group that showed the highest frequency in the present study could not be compared to previous data, since each study has used different designs. Recently, the frequency of *C. difficile* infection in children has been reported to be increasing (27-31).

There are insufficient data regarding colonization rate of *C. difficile* in Iran. Jalali et al., (32) extracted *C. difficile* isolates from 19/89 (21%) patients. In our study, the colonization rate with *C. difficile* among stool specimens from Ilamian children was found to be 10% (8/80). Our finding is relatively comparable with the study performed by Kouhsari et al. (4), where *C. difficile* was identified in 14% (35/250) of hospitalized patients, while in conflict with a previous Iranian study (2002-2006) that reported a lower CDI incidence (6.1%, 57/942) (33). The incidence of *tcdA*$^-$/*tcdB*$^+$ *C. difficile* strains is extensively increasing and ranges from 3% to 92% worldwide, however, the clinical characteristics of these strains have not been well recognized (34). The prevalence of *tcdA*$^-$/*tcdB*$^+$ strains varies depending on the geographic region being studied. In a study conducted in Iran, the prevalence of *tcdA*$^-$/*tcdB*$^+$ strains was 8% (35). In Europe, 6.2% of *C. difficile* isolates were *tcdA*$^-$/*tcdB*$^+$ variant (36). In this study, 37.5% (3/8) were detected as *tcdA*$^-$/*tcdB*$^+$ *C. difficile* strains, and all of patients containing *tcdA*$^-$/*tcdB*$^+$ *C. difficile* were symptomatic. All pediatric isolates were negative for binary toxin genes.

Among healthy infants aged < 1 month, carriage with *C. difficile* was an average rate of 37% among individuals. In infants aged 1- 6 months, colonization decreased to an average rate of 30%. This trend continued, with recovery dropping to 14% among 6-12 months kujkkkminfants, and 10% in children aged >1 year (15, 16). It is demonstrated that age effectively impacts on the colonization rate of *C. difficile* among symptomatic and asymptomatic children (11, 13, 26-28). Our findings demonstrated that the carriage rate in children aged < 3 years was much higher than in children aged > 3 years. These results are relatively in accordance with those of previous reports in European countries, USA and Japan, where the carriage rates in children aged < 2 years were found to be high (37-42). It has been suggested

that the gut microbiota could inhibit the colonization or growth of *C. difficile* in older infants (43, 44). Mostly, *C. difficile* colonization in neonates is associated with environmental contamination rather than maternal transmission (12, 15, 26, 29, 45-47). A broad range of *C. difficile* colonization (2-71%) in hospitalized neonates has been reported in previous surveys from other countries (12, 40, 47). A Japanese study reported that the intestinal-carriage rate of *C. difficile* was 61% (41/67) in asymptomatic hospitalized neonates (48). Pulsed field gel electrophoresis (PFGE) proved that 96.3% (53/55) of isolates were identical (48). Our findings showed that all the intestinal-carriage rate of *C. difficile* was 0% in asymptomatic hospitalized neonates.

We detected *C. difficile* strains using PCR amplification of rRNA intergenic spacer (ITS) regions (PCR ribotyping), due to their discriminatory power, reproducibility, ease of performance, and cost effectiveness (18, 21). In line with another study (32) in Iran, we have disclosed 8 unique RTs of 8 *C. difficile* dispersed in Ilamian pediatric population at Imam Khomeini hospital in Ilam, Iran. The dissemination of RTs diverges from region to region. Generally, in most European countries and North America, RT027 have been reported more than others (49). The main RTs in Asia were indicated to be 017 and 018 (3). There is infrequent data about the most dominant RTs of *C. difficile* strains in the Middle East (50, 51). In Iran, few studies were performed on the PCR-ribotyping of *C. difficile* strains, therefore, it is not promising to compare our findings with previous reports due to incoherence or the lack of molecular typing methods (18). In this study, three toxigenic isolates (*tcdA*$^-$/*tcdB*$^+$) belonged to PCR RTs of R2/R3/R5. Our finding necessitates the establishment of more epidemiological surveys in neonates. Nevertheless, there were some limitations in our study including the small sample size and shortage of financial sources. Hence, for future studies we recommend that larger sample size be obtained.

Conclusions

The carriage rate of *C. difficile* was 10%. The colonization rate in children agedl < 1 - 3 years was much higher than in children > 3 years old. Thirty-seven-five percent of isolates were determined as community-associated colonization. Our findings showed that *C. difficile* isolates were genetically diverse. Thus, more accurate risk stratification, further large-scale prospective and other screening/surveillance modalities are required to monitor the varying epidemiology and role of *C. difficile* in Iranian children.

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Publication and authorship
Mahshad Sayyadi and Gholamreza Kalvandi contributed to the conception and design of the work. Hossein Kazemian and Nourkhoda Sadeghifard contributed to design of the work, and final approval of the version to be published. Ebrahim Kouhsari and Zahra Ghafouri contributed in drafting the work, native speaker English editing and revising it critically for important intellectual content. Nourkhoda Sadeghifard contributed in revising the article and final approval of the version to be published.

Riassunto

Caratteristiche della colonizzazione da *Clostridioides difficile* ***nei bambini di Ilam, Iran***

Premessa. Se è noto il progressivo incremento della colonizzazione da parte del *Clostridioides difficile* della popolazione pediatrica durante la degenza ospedaliera,

la prevalenza del fenomeno in Iran è tuttora bisognoso di studio.

Obiettivo. Scopo del presente studio è stato di determinare, tra il 22 Maggio ed il 22 Settembre 2018, la frequenza della colonizzazione intestinale da *C. difficile* della popolazione pediatrica di Ilam e di effettuare la caratterizzazione molecolare dei ceppi isolati di quel microorganismo.

Materiali e metodi. Da 40 bambini d'età 0-5 anni sono stati ottenuti 80 tamponi, di cui 40 all'atto del ricovero (tempo 0) e 40 dopo 5 giorni di degenza: i primi per valutare la colonizzazione comunitaria, gli altri per valutare la colonizzazione successiva al ricovero. In tutti i campioni è stata ricercata la presenza di *C difficile*, e nei ceppi isolati è stata ricercata la produzione delle tossine clostridiali A/B, seguita dalla caratterizzazione molecolare.

Risultati. La colonizzazione globale da *C difficile* è risultata pari al 10% (8/80), quella di *C difficile* tossigeno pari al 3,75% (3/80). Sulla base dell'età, la frequenza di carriage è del 37,5%, del 50% e del 12,5% alle età, rispettivamente, di <1, 1-3 e 3-5 anni, documentando così l'assoluta prevalenza di colonizzazione nei soggetti in età inferiore a 3 anni. Abbiamo identificato in tutto 8 ribotipi diversi di *C difficile*. Il 37.5% degli isolamenti (3/8) è stato identificato come di origine comunitaria ed appartenevano ai ribotipi 7, 8 e 9.

Conclusioni. I nostri risultati meritano di essere confermati da più ampi studi. Comunque, dato che un terzo dei casi era di origine comunitaria, le stime dell'incidenza dell'infezione da *C difficile*, che include solo i bambini ospedalizzati, potrebbe sottostimare in senso il vero burden della malattia nei bambini.

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