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DETECTION, RIBOTYPING AND ANTIMICROBIAL RESISTANCE PROPERTIES OF CLOSTRIDIUM DIFFICILE STRAINS ISOLATED FROM THE CASES OF DIARRHEA

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ABSTRACT
Background: *Clostridium difficile* is the most prevalent cause of antibiotic-associated infectious diarrhea al-around the world. Prevalence of virulent and resistant strains of *Clostridium difficile* is increasing now a day. The present investigation was carried out to study the prevalence, ribotyping and antibiotic resistance pattern of *C. difficile* isolated from diarrheic and non-diarrheic pediatrics. **Materials and methods:** Four-hundred stool specimens were collected from the diarrheic and non-diarrheic pediatrics hospitalized due to the diseases other than diarrhea. Samples were cultured and their positive results were subjected to disk diffusion and PCR-based ribotyping. **Results:** Thirty-five out of 400 (8.75%) samples were positive for *C. difficile*. Prevalence of *C. difficile* in diarrheic and non-diarrheic pediatrics were 11.25% and 4.16%, respectively. Male had the higher prevalence of bacteria than female ($P < 0.05$). eight to twelve months old pediatrics were the most commonly infected group. R27 (14.28%), R1 (10.71%), R12 (7.14%), R13 (7.14%) and R18 (7.14%) were most commonly detected ribotypes. There were no positive results for studied ribotypes in non-diarrheic pediatrics. *C. difficile* strains had the highest levels of resistance against tetracycline (71.42%), erythromycin (57.14%), moxifloxacin (48.57%), metronidazole (28.57%) and clindamycin (22.85%) antibiotics. **Conclusion:** Prescription of antibiotics in diarrheic pediatrics, males and also 8-12 months old pediatrics should be done in a regular and cautious manner.
Key words: *Clostridium difficile*, Diarrhea, Pediatrics, Ribotyping, Antimicrobial resistance.

1. INTRODUCTION

Infectious diarrhea is one of the most critical diseases among children especially in developing countries. Documented data revealed that there are about two billion cases of diarrhea in adults and children and 1.9 million cases of diarrhea in children worldwide every year (1). The mortality rate of infectious diarrhea in children is high and more than 5000 children are dying (1). Therefore, specific attention should be done to identify new aspects of infectious diarrhea in children.

Clostridium difficile is an important cause of antibiotic-associated diarrhea and one of the most common healthcare-associated infections al-around the world (2, 3). *C. difficile* is a spore-forming, anaerobic, gram-positive bacillus bacterium which causes a wide spectrum of illnesses from asymptomatic colonization or mild diarrhea to fulminant disease characterized by toxic megacolon, sepsis, pseudomembranous colitis and death (2-4). *C. difficile* infection is less common in children than adults, but its prevalence is increasing in children (5-7). Disease is mainly occurred in children who have been treated with long term antibiotic therapy (8, 9).

PCR ribotyping of *C. difficile* isolates of human clinical samples is progressively being employed for molecular epidemiological analysis. It is based on the presence of some alleles of the rRNA operon on the bacterial chromosome differing by the length of the intergenic spacer region located between the 16S and the *23S rRNA* genes (10). PCR based ribotyping can be used for inter laboratory comparison and creation of reference library (10). In humans approximately 300 PCR ribotypes are recognized in *C. difficile* of human clinical infections and the most prevalent in many research studies is PCR ribotype 027 (RT027) (11-13). Data showed that the diarrheal diseases caused by 027 ribotype of *C. difficile* are more severe, with higher rates of morbidity, mortality

and antibiotic resistance (11-13).

Treatment is the most critical aspect to control expansion of C. *difficile*-based diarrhea in children. High prevalence of antibiotic resistance in the C. *difficile* strains of clinical infections cause more severe diseases for longer periods of time (14-16). According to the recent epidemiological studies, C. *difficile* strains of clinical samples represented a high prevalence of resistance (20-80%) against commonly used antibiotics (14-17). Therefore, it is important to know exact antibiotic resistance pattern of C. *difficile* strains of diarrhea to found the best therapeutic approach for control and even establishment of novel therapeutic ways for treatment of C. *difficile*-based diarrhea in children.

According to the lack of epidemiological and microbiological data on the prevalence of C. *difficile* in children, the present investigation was carried out to study the prevalence, ribotyping and antibiotic resistance properties of C. *difficile* strains isolated from the stool samples pf diarrheic and non-diarrheic pediatrics patients.

2. MATERIALS AND METHODS

Ethical issues

This research project was approved by the ethical committee of Education Hospitals of Tehran, Iran (Reference number: ETHC 2015234PO). Written informed consent was obtained from the patients or their parents.

Samples collection and bacterial isolation

From March to October 2015, a total of 400 fecal specimens were collected from the diarrheic (n=180) and non-diarrheic (n=220) hospitalized pediatrics (male and female with various ages) who were referred for diseases other than diarrhea such as pneumonia, urinary tract infection, skin infections and acute respiratory illness. All diarrheic pediatrics showed clinical signs of nosocomial diarrhea (diarrhea occurring more than 72 hours after admission to hospital for non-diarrheal causes). All samples were immediately transferred to the laboratory in cooler with ice-packs using the Cary-Blair transitional media (Merck, Germany).

Five grams of each specimen was transferred to 20 mL of *C. difficile* broth (Merck, Germany) containing 40 g/l proteose peptone, 5.0 g/l disodium hydrogen phosphate, 0.1 g/l magnesium sulphate, 2.0 g/l sodium chloride, 6.0 g/l fructose and 1.0 g/l sodium taurocholate supplement with *C. difficile* selective supplement (Merck, Germany) and 5% (v/v) defibrinated sheep blood. After incubation at 37°C for 2 days under anaerobic conditions, 2 mL of the enrichment broth was added to 2 mL of 96% ethanol in a centrifuge tube and homogenized for 50 min on a shaker at room temperature. After centrifugation (3800 × g for 10 min), a loopful of the sediment was streaked onto Cycloserine Cefoxitin Fructose Agar (CCFA, Merck, Germany) containing sodium taurocholate. All plates were incubated in an anaerobic chamber (Don Whitley Scientific Ltd., Shipley West Yorkshire, UK) at 37°C for 48 h. Colonies of *C. difficile* were identified on the basis of their characteristic colony morphology (yellow, ground glass appearance), Gram staining, odour (horse dung smell), their chartreuse fluorescence under long-wave UV light (~360 nm) and L-proline aminopeptidase test (18).

Antimicrobial resistance properties of bacterial isolates

Antimicrobial susceptibility tests were performed by the Kirby–Bauer disc diffusion method using Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084), according to the Clinical and Laboratory Standards Institute guidelines (19). After incubating the inoculated plate anaerobically at 37 °C for 2 days, the susceptibility of the *C. difficile* isolates against metronidazole (5 µg/disk), rifampicin (5 µg/disk), vancomycin (5 µg/disk), clindamycin (2 µg/disk), erythromycin (15 µg/disk), fidaxomicin (5 µg/disk), moxifloxacin (5 µg/disk), tigecycline (15 µg/disk), linezolid (30 µg/disk), fusidic acid (10 µg/disk) and tetracycline (30 µg/disk) antimicrobial agents (Oxoid, UK) was measured. Results were interpreted in accordance with interpretive criteria provided by CLSI (2012) (19). *C. difficile* ATCC 9689 was used as quality control organisms in antimicrobial susceptibility determination.

DNA extraction

Genomic DNA was extracted from typical colonies of *C. difficile* using genomic DNA extraction Kit (Fermentas, Germany) according to the manufacturer's recommendation. The isolated DNA was quantified by spectrophotometric measurement at a wave length of 260 nm according to the method described by Sambrook and Russell (20). The extracted DNA of each specimen was kept frozen at -20°C until used.

PCR ribotyping

Ribotyping was done using a PCR technique. A programmable DNA thermo-cycler device (Eppendrof Mastercycler 5330; Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions. PCR reaction was performed in a total volume of 25 µL including 0.5 ml tubes containing 1 µg of genomic DNA, 1 µM of each primer (p3-F: 5′-CTGGGGTGAAGTCGTAACAAGG-3′ (position 1445 to 1466 of the *16S rRNA* gene) and p5-R: 5′-GCGCCCTTTGTAGCTTGACC-3′ (position 20 to 1 of the *23S rRNA* gene) (21), 2 mM $Mgcl_2$, 200 Mm dNTP, 2.5 µL of 10X PCR buffer and 1 unit of Taq DNA polymerase (Fermentas, Germany). PCR cycles consisted of an initial denaturation step (95°C for 5 min) followed by 30 amplification cycles (denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 1 min) with a final elongation at 72°C for 5 min and amplified samples were held at 4°C. *C. difficile* 027/NAP1/BI strain was used as a positive control. A negative-DNA control was performed by adding one µL of sterile ultrapure deionized water.

Analysis of PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H_2O and stir to dissolve). Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder of Fermentas, Germany). After electrophoresis, the amplicons were visualized with ultraviolet light after ethidium bromide (5 $\mu g.mL^{-1}$) staining and photographs were obtained in UVIdoc gel documentation systems (UK).

Ribotyping patterns

Comparison of PCR ribotyping patterns was performed

visually. All isolates were typed using the PCR ribotyping method (22) and Gel Compare software. Strains with ribotype patterns that differed by at least one band were assigned to different types. Ribotype group was designated by upper and lower-case letters combined with a number.

Statistical analysis

All data were analyzed using *SPSS* software (Version 19. SPSS Inc, United States) with respect to the chi-square and fisher exact tests to find any significant correlation between incidence of *C. difficile*, its ribotypes and antibiotic resistance. Statistical significance was regarded at a P value < 0.05. Investigation of ribotypes of *C. difficile* were performed using the Gel Compar software.

3. RESULTS

Table 1 represents the total prevalence of *C. difficile* in the stool samples of diarrheic and non-diarrheic pediatrics. Results showed that 35 out of 400 (8.75%) stool samples were positive for *C. difficile*. Total prevalence of *C. difficile* in diarrheic and non-diarrheic pediatrics were 11.25% and 4.16%, respectively. Statistically significant differences were seen between the prevalence of *C. difficile* and types of stool samples (diarrheic and non-diarrheic) ($P < 0.01$), prevalence of *C. difficile* and age of pediatrics ($P < 0.05$) and finally between prevalence of *C. difficile* and sex of patients ($P < 0.05$). On the other hand, *C. difficile* had the higher prevalence in

Types of samples			No. samples collected	Prevalence of C. difficile (%)
Diarrheic pediatrics	Male	4-8 months	30	5 (16.66)
		8-12 months	21	7 (33.33)
		12-16 months	26	5 (19.23)
		16-20 months	23	2 (8.69)
		Total	100	19 (19)
	Female	4-8 months	23	2 (8.69)
		8-12 months	17	4 (23.52)
		12-16 months	22	2 (16.66)
		16-20 months	18	1 (5.55)
		Total	80	9 (11.25)
	Total		180	28 (15.55)
Non-diarrheic pediatrics	Male	4-8 months	33	1 (3.03)
		8-12 months	26	3 (11.53)
		12-16 months	35	1 (2.85)
		16-20 months	25	-
		Total	120	5 (4.16)
	Female	4-8 months	21	-
		8-12 months	29	2 (6.89)
		12-16 months	27	-
		16-20 months	23	-
		Total	100	2 (2)
	Total		220	7 (3.18)
Total			400	35 (8.75)

Table 1. Total prevalence of C. difficile in the diarrheic and non-diarrheic pediatrics.

male and 8-12 months pediatrics in both studied groups.

Figure 1 represents the results of the gel electrophoresis of PCR products for various ribotypes of *C. difficile* of studied samples. Table 2 shows the ribotyping pattern of *C. difficile* isolates of diarrheic and non-diarrheic pediatrics. There were no positive results for all studied ribotypyes in

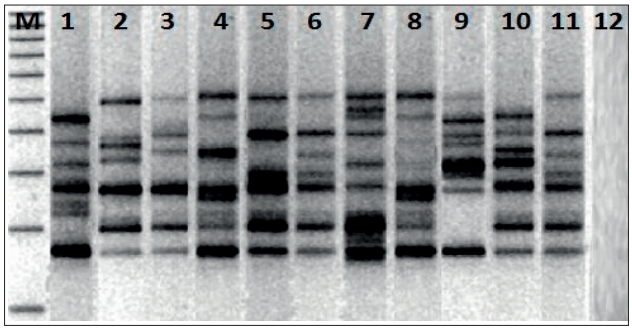

Figure 1. Gel electrophoresis of PCR for detection of ribotypes of C. difficile isolated from diarrheic and non-diarrheic pediatrics (M: 100 bp DNA ladder (Fermentas, Germany), 1-11: R7, R27, R83, R87, R29, R31, R15, R1, R82, R8, R88 ribotypes of C. difficile, respectively and line 12: negative control.

Ribotypes	Distribution (%)	
	Diarrheicchildren(28*)	Non-diarrheic children (7*)
R1	3 (10.71)	-
R7	1 (3.57)	-
R8	1 (3.57)	-
R12	2 (7.14)	-
R13	2 (7.14)	-
R15	1 (3.57)	-
R18	2 (7.14)	-
R27	4 (14.28)	-
R28	1 (3.57)	-
R29	1 (3.57)	-
R31	1 (3.57)	-
R32	1 (3.57)	-
R81	1 (3.57)	-
R82	1 (3.57)	-
R83	1 (3.57)	-
R86	1 (3.57)	-
R87	1 (3.57)	-
R88	1 (3.57)	-
Other ribotypes	2 (7.14)	7 (100)
Total	28 (100)	7 (100)

Table 2. Ribotyping pattern of C. difficile isolated from the diarrheic and non-diarrheic pediatrics.. *Number of positive strains.

the non-diarrheic pediatrics. The most commonly detected ribotypes in the group of diarrheic pediatrics were R27 (14.28%), R1 (10.71%), R12 (7.14%), R13 (7.14%) and R18 (7.14%). Totally, 7.14% of *C. difficile* ribotypes of diarrheic pediatrics were determined as other ribotypes (Those that were not detected using our technique).

Table 3 represents the total prevalence of antibiotic resistance in diarrheic and non-diarrheic pediatric patients. Bacterial strains of our investigation harbored the highest levels of resistance against tetracycline (71.42%), erythromycin (57.14%), moxifloxacin (48.57%), metronidazole (28.57%) and clindamycin (22.85%), while resistance against fidaxomicin (8.57%), linezolid (8.57%) and fusidic acid (11.42%) were low. Statistically significant difference was seen between the types of samples and prevalence of antibiotic resistance ($P < 0.05$). In the other hand, diarrheic pediatrics had the higher prevalence of antibiotic resistance than non-diarrheic ($P < 0.05$).

Types of samples (No. positive)	Antibiotic resistance pattern (%)										
	Met	Rif	Van	Clin	ERT	FDX	Mox	Tig	Lin	Fus	Tet
Diarrheic pediatrics (28)	9 (32.14)	5 (17.85)	6 (21.42)	7 (25)	17 (60.71)	3 (10.71)	15 (53.57)	5 (17.85)	3 (10.71)	4 (14.28)	21 (75)
Non-diarrheic pediatrics (7)	1 (14.28)	-	1 (14.28)	1 (14.28)	3 (42.85)	-	2 (28.57)	-	-	-	4 (57.14)
Total (35)	10 (28.57)	5 (14.28)	7 (20)	8 (22.85)	20 (57.14)	3 (8.57)	17 (48.57)	5 (14.28)	3 (8.57)	4 (11.42)	25 (71.42)

Table 3. Antimicrobial resistance properties of Clostridium difficile strains of diarrheic and non-diarrheic pediatric patients.. *In this table Met: metronidazole (5 µg/disk), Rif: rifampicin (5 µg/disk), Van: vancomycin (5 µg/disk), Clin: clindamycin (2 µg/disk), ERT: erythromycin (15 µg/disk), FDX: fidaxomicin (5 µg/disk), Mox: moxifloxacin (5 µg/disk), Tig: tigecycline (15 µg/disk), Lin: linezolid (30 µg/disk), Fus: fusidic acid (10 µg/disk), Tet: tetracycline (30 µg/disk).

4. DISCUSSION

As far as we know, the present investigation is the first prevalence report of ribotyping and antibiotic resistance properties of *C. difficile* infections in diarrheic and non-diarrheic pediatric patients of various ages and sexes in Iran. We found that 8.75% of all studied stool samples, 11.25% of diarrheic stool samples and 4.16% of non-diarrheic stool samples were positive for *C. difficile*. We also found that diarrheic pediatrics harbored higher resistant and also virulent strains of this bacterium.

Unfortunately, the impact of this bacterium as a main cause of antibiotic-associated diarrhea especially in children less than 5 years old has been ignored. Studies which have been done in this field in Iran are scarce. Khoshdel et al. (2015) (23) revealed that 52% of diarrheic and non-diarrheic stool samples of Iranian children were positive for *C. difficile* which was entirely higher than our results. They showed that boys and 13 to 24-month age children had the higher prevalence of *C. difficile*. They also revealed that the most commonly detected ribotypes in the *C. difficile* isolates of hospitalized children were RT027 (11.52%), RT01 (9.61%) and RT013 (7.68%) which was similar with our findings. Another Iranian investigation showed that a total of 942 stool samples from patients with nosocomial diarrhea were tested and 57 samples (6.1%) were positive for toxigenic *C. difficile* (24). Keesen et al. (2013) (25) reported that *C. difficile* had a high prevalence of human-based clinical samples like diarrhea. They mentioned that 37% of the isolates were resistant to four or more antimicrobial agents. The majority of human isolates were susceptible to amoxicillin (100%), tetracycline (100%) and clindamycin (96%) and resistant to ciprofloxacin (96%). High levels of resistance against erythromycin and moxifloxacin had also been reported. Previous investigation which conducted on the United States (26) reported that of the 29 PCR ribotypes identified in the cases of diarrhea, the 027 ribotype was the most commonly detected (28.1%). They revealed that clindamycin and moxifloxacin resistances (36.8% and 35.8%, respectively) were the most frequent resistance phenotypes observed. Reduced susceptibility to vancomycin was observed in 39.1% of 027 isolates. All of their findings were similar to our results. Similar results have been reported by Hecht et al. (2007) (Maywood) (27), Pelaez et al. (2002) (Spain) (28), Dong et al. (2013) (China) (29), Goudarzi et al. (2013) (Iran) (30), Rao et al. (2015) (Nashville) (31) and MacCannell et al. (2006) (Canada) (32).

We found that bacterial strains of our survey had the highest levels of resistance against tetracycline, erythromycin and moxifloxacin. During the rapid emergence of type 027 a decade ago, resistance of type 027 against tetracycline, erythromycin and moxifloxacin was frequent (33, 34), which was also in line with the association of type 027 and fluoroquinolones in epidemiological studies. According to the high and irregular prescription of antibiotics especially tetracycline, erythromycin, moxifloxacin, metronidazole and clindamycin in Iranian hospitals and health cares, it is not surprising that the *C. difficile* strains of pediatrics (that might be the first time that take antibiotics) had such high levels of antibiotic resistance. As far as we know, the present investigation reported one of the highest prevalence of 027 ribotype and antibiotic resistance in the world. High differences which were reported in the prevalence rate and also levels of antibiotic resistance in different studies maybe due to the differences in the types of samples, method of sampling, method of experiment, types of ribotypes and even availability of antibiotics for prescription in various studies.

5. CONCLUSION

In conclusion, our results reported the high prevalence of *C. difficile*, 027 ribotype and high levels of resistance against tetracycline, erythromycin, moxifloxacin, metronidazole and clindamycin antibiotics. Prevalence of all studied factors in diarrheic pediatrics, males and also 8-12 months old pediatrics were higher than other groups. It seems that prescription of antibiotics in these groups of pediatrics should be done in a highly regular and careful manner. Clinician should pay more attention in prescription of antibiotics in the cases of diarrhea especially in high risk groups of children.

• Conflict of interest: none declared.

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