JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Detection of *Clostridium difficile* among diarrheic children using cultural and polymerase chain reaction technique

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Abstract

Introduction: *Clostridium difficile* is the most common cause of antibiotic-associated diarrhea and colitis. Several methods are available for the detection of *C. difficile* in stool samples. This study aimed to use glutamate dehydrogenase (GDH), toxin detection, culture and polymerase chain reaction (PCR) techniques for the diagnosis of this pathogen.

Methodology: A total of 300 stool samples were collected from children with hospital acquired diarrhea (HA-D), community acquired diarrhea (CA-D), and hospitalized non-diarrheic children as control with ages ranging from 6 months to 6 years (mean 3.7 ± 1.7). Each stool sample was divided into two parts; one part was tested for the enzyme GDH, toxin A and B and then cultured on selective media; and the other part for direct DNA extraction.

Results: From a total of 300 stool samples, 9 (3.0%) were positive for *C. difficile* by the PCR technique, 7 (7%) samples of which were from HA-D cases and 2 (2.0%) from CA-D cases; the control group samples were negative. The enzyme GDH was detected in 12 (12%) samples and toxins A and B in 8 (8%) samples from HA-D cases compared to 5 (5%) and 2 (2%), respectively from CA-D cases. Both GDH and the toxins were negative in control samples. Only 19 (19.0%) samples from HA-D cases gave suspected growth and all of these were negative by PCR.

Conclusions: Based on the results of this study, we conclude that the PCR technique is the only reliable method for the diagnosis of this pathogen.

Key words: *Clostridium difficile*, diarrhea, culture, PCR.

J Infect Dev Ctries 2023; 17(10):1452-1457. doi:10.3855/jidc.17473

(Received 30 September 2022 – Accepted 05 April 2023)

Introduction

Clostridium difficile is a motile, rod-shaped, Gram-positive bacterium, which is known to be a leading cause of antibiotic-associated diarrhea, especially nosocomial infections [1]. The organism is considered as one of the most frequent causes of nosocomial infections [2,3] and is associated with a wide range of infections including a self-limited antibiotic-associated diarrhea (AAD), antibiotic-associated colitits (AAC), and serious conditions like pseudomembranous colitis and toxic megacolon [4]. *C. difficile* flourishes and colonizes the human intestinal tract after the gut flora has been altered by antibiotic therapy, especially with oral broad-spectrum antibiotics [5]. Two large toxins, tcdA enterotoxin and tcdB cytotoxins (308 kDa and 270 kDa, respectively), are recognized as the main virulence factors of *C. difficile*, disrupting the tight junctions of the intestinal epithelial cells and resulting in inflammation and increased permeability of the intestine [6]. Both the *tcdA* and *tcdB* genes are part of the PaLoc operon, which also contains *tcdR*, *tcdE*, and *tcdC*, of which *tcdC* is a putative negative regulator of *tcdA* and *tcdB* [7]. Antibiotic therapy is not only a risk factor for the development of *C. difficile* infection (CDI), but studies have also shown that the presence of antibiotics in the gut can increase toxin production [8-10], germination, and the expression of colonization factors [11,12] within the gut. *C. difficile* infection results in a wide range of symptoms, including fever, abdominal pain, mild diarrhea, and pseudomembranous colitis. The hypervirulent strains that are resistant to the current therapy can produce high titers of toxins and pose a challenge to the treatment of the infection worldwide [13]. Several methods have been proposed by researchers for the detection of *C. difficile* in stool samples, such as isolation using selective culture media, antigen-based method, toxin detection methods, as well as direct nucleic acid amplification [14-18]. To the best of our knowledge, there is no existing data concerning the prevalence of this pathogen in Duhok province/Iraq.

Therefore, the main objectives of the current study were to find the prevalence of *C. difficile* among nosocomial and community diarrheic children using both cultural and molecular methods, to study the antibiogram of the isolated strains, and to evaluate the association of the risk factors with the development of *C. difficile* infection.

Methodology

Sample collection

This cross-sectional study was performed in Hivi Pediatric Teaching hospital in Duhok city, Iraq from October 2021 to the end of May 2022. Inclusion criteria for hospital acquired diarrhea (HA-D) included (1) Patients admitted to hospital for other causes rather than diarrhea, (2) Patients received antibiotic therapy after hospitalization and developed diarrhea within 48 to 72 hours, (3) Patients had to produce at least 3 unformed stools over a period of 24 hours. For community acquired diarrhea (CA-D), any patients hospitalized for diarrhea were enrolled in the study. Patients whose diarrhea was due to other proven causes were excluded from the study. A total of 300 stool samples were collected from children with HA-D (100 samples), CA-D (100 samples) and hospitalized non diarrheic children (100 sample) as control with ages ranging from 6 months to 6 years with a mean of 3.7 ± 1.7 years. Consent was obtained from the children's guardians and a special questionnaire was designed to collect information on the gender, age, residence, and antibiotics and proton pump inhibitors received in order to correlate with *C. difficile* infection. Stool samples were collected in clean and dry screw capped plastic containers and transferred immediately in a cool box to the microbiology research laboratory of the Department of Biology, College of Science, University of Duhok. Each stool sample was divided into two parts, one part for isolation and the other for direct DNA extraction for polymerase chain reaction (PCR) technique.

Samples

All stool samples were divided into two parts, one part was screened for both glutamate dehydrogenase (GDH) enzyme and toxin A and B, and then cultured on selective culture media under anaerobic conditions, while the other part was subjected to direct DNA extraction for PCR.

Glutamate dehydrogenase test

Before isolation, all stool samples were screened for GDH enzyme using GDH immunochromatography strip test kit (CTK Biotech, Madrid, Spain) according to the instructions supplied by the kit [5].

C. difficile toxin A and B test

Each stool sample was tested for toxin A and B using *C. difficile* toxin A and B immunochromatography strip test kit (CTK Biotech, Madrid, Spain) following the instructions supplied by the kit [5].

Bacterial isolation

Before culturing, each stool sample was subjected to alcohol shock in which stool was mixed with an equal volume of absolute ethyl alcohol and incubated at room temperature (20-25 ℃) for 1 hour, and then centrifuged at 5000 rpm for five minutes. The suspension was vortexed again and a loop full of suspension was plated on cycloserine-cefoxitin-fructose agar (CCFA) (Candalab, Madrid, Spain) with the supplement (SIGMA-Aldrich, Massachusetts, USA) and 7% horse blood. The plates were incubated in an anaerobic jar using anaerobic gas packs (Gas Pack Anaerocult® A Merk Darmstadt, Germany) for 72 h at 37 °C [19]. Identification was based on colony morphology, Gram staining, spore stain and the final diagnosis was made by PCR.

DNA extraction from suspected bacterial colonies

DNA was extracted from colonies of anaerobic Gram-positive spore-forming bacilli using the Dneasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany).

DNA extraction from stool samples

DNA was extracted from 300 stool samples (100 samples from HA-D, 100 samples from CA-D and 100 samples from hospitalized non-diarrheic children as a control). All the samples were treated with stool transport and recovery buffer (S.T.A.R, Roche, Mannheim, Germany) to stabilize nucleic acids and inactivate stool sample inhibitors, and processed with a High Pure PCR Template Preparation kit for DNA extraction (Roche, Mannheim, Germany) [20]. The purity and concentrations of extracted DNA were checked by a Nanodrop machine (DeNOVIX, Wilmington, USA), in which the purity of DNA ranged from 1.95 to 2.02 ng/μL at wavelength 260/280 and concentrations ranged from 120.32 to 256.51 ng/mL.

Identification of Clostridium difficile by PCR technique

The extracted DNA was screened for the presence of *C. difficile* via direct detection of *C. difficile* 16S

Table 1. Results of detection of *C. difficile* by phenotypic and molecular methods.

Source of stool samples	Number	GDH +ve (%)	Toxin A & B +ve (%)	Culture	PCR +ve
HA-D	100	12 (12.0)	8 (8.0)	19 (19.0)	7 (7.0)
CA-D	100	5 (5.0)	2 (2.0)	0.(0.0)	2 (2.0)
Control	100	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

GDH: Glutamate dehydrogenase enzyme; PCR: Polymerase chain reaction.

rDNA using the following primers: CIDIF-F CTT GAA TAT CAA AGG TGA GCC A and CIDIF-R CTA CAA TCC GAA CTG AGA GTA (Eurofins, Ebersberg, Germany) [21]. The PCR reaction was carried out in a final volume 30 µL where 3 µL of DNA template, 1 µL of each primer, 15 µL of green master mix (Roche, Mannheim, Germany) and 10.0 µL of nuclease free water were included in each reaction. The thermal profile of the PCR reaction was as follows: initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 5 min in a thermocycler (Applied Biosystem version 9700, California, USA). Afterwards, the amplicons were separated with agarose gel electrophoresis (BioRad, Hercules, USA) and photographed to visualize the specific band at 1085 bp. The control positive was DNA of DSMZ 1296 strain kindly provided by the Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Hannover, Germany.

Ethical approval

Ethical approval was obtained from the Research Ethics Committee of Duhok Directorate General Health No.13072021-7-7.

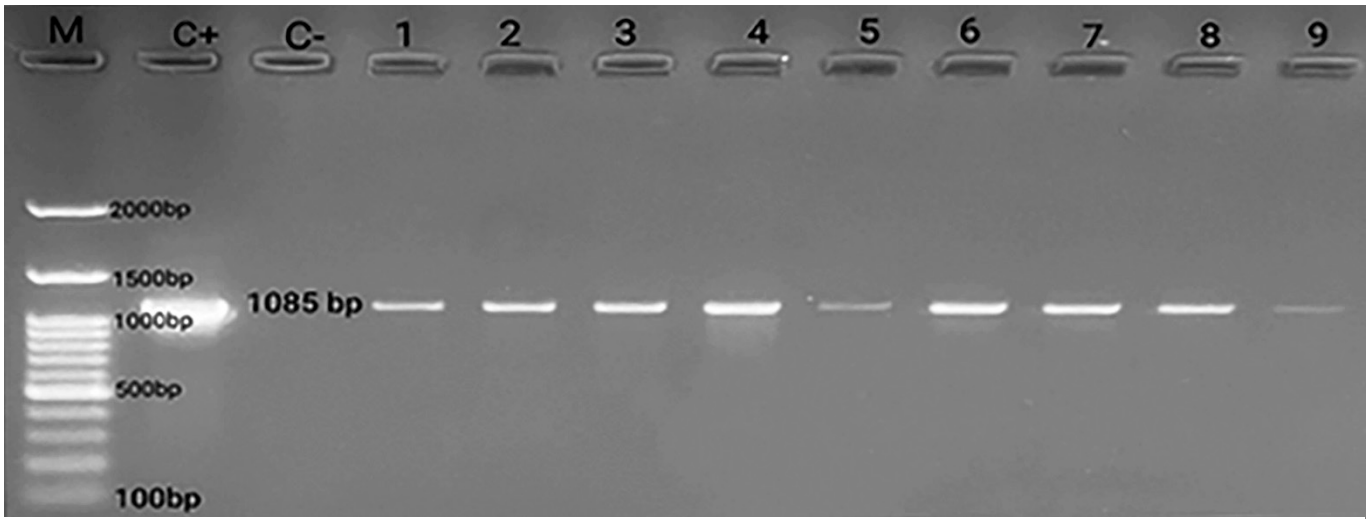
Statistical analysis

The data were analyzed using R-statistics version 4.2. The confidence level was set at 95 % and p value < 0.05 was considered significant.

Results

From a total of 300 stool samples collected from children with HA-D (100 samples), CA-D (100 samples), and non-diarrheic children (100 samples) who were admitted to hospital for other illnesses, nine (3.0%) samples were positive for *C. difficile* when tested by PCR, 7 (7%) of which were from HA-D, 2 (2.0%) from CA-D, while all of the control group samples were negative as shown in Figure 1. Among the HA-D cases, 12 (12%) samples were positive for the enzyme GDH and 8 (8%) were positive for both toxin A and B compared to 5 (5%) samples for the enzyme GDH and 2 (2%) samples for both toxin A and B among CA-D cases. Only 19 out of 20 samples positive for GDH and toxins from HA-D cases were grown on selective media and showed Gram-positive spore-forming bacilli; however, all of them were negative when tested with PCR. No growth was obtained from 7 samples of CA-D which were positive for GDH and toxins as well as from the rest of the samples as shown in Table 1. Among 12 positive samples for GDH in HA-

Figure 1. Detection of Clostridium difficile from stool samples using species-specific 16S rRNA primers.



Electrophoresis was performed on 1.2% agarose gel at 70V for 1 hour running at 5-8v/cm. Lane M contained DNA molecular weight marker (2000 bp); Lane C+: Positive control was DNA of DSMZ 1296 strain and NTC: Non-template control. Numbfigureers from 1-9 represent samples.

Table 2. Results of PCR and immunochromatography strip methods for diagnosis of *C. difficile.*

Sample	Total No.	GDH		Toxin A & B	
		No. (%) of positive by strip method	No. (%) of positive by PCR	No. (%) of positive by strip method	No. (%) of positive by PCR
HA-D	100	12 (12.0)	7 (7.0)	8 (8.0)	7 (7.0)
CA-D	100	5 (5.0)	2 (2.0)	2 (2.0)	2 (2.0)
Control	100	0	0	0	0

GDH: Glutamate dehydrogenase enzyme; PCR: Polymerase chain reaction.

D cases, 7 (58.3%) were positive by PCR technique, while 2 (40%) samples from five GDH positive samples among CA-D cases were positive by PCR. For toxin A and B, 7 (87.5%) of 8 samples were positive by PCR among HA-D cases compared to 2 of 2 (100%) toxin positive samples in CA-D cases as shown in Table 1. All samples from the control group were negative for both GDH and toxin A and B. For toxin A and B, all positive cases were positive for both toxin A and B and no case was found to be positive for one toxin. There were positive samples for GDH and toxin A and B but also negative ones when tested by PCR.

A high percentage (10.5 %) of *C. difficile* was detected among the age group 4-5 years and the lowest percentage (5.5 %) was among the age group 2-3 years of age, while it was not detected at all in the age group 4-5 years who had developed HA-D as shown in Table 2. No significant correlation was found between *C. difficile* infection and HA-D age groups ($p < 0.85$).

Among CA-D cases, similar percentages (5.8% and 5.2%) of *C. difficile* were recorded for the age groups 1-2 years and 3-4 years, respectively as shown in Table 3. No significant relation was found between the *C. difficile* infection and age group ($p < 0.6$).

Similar percentages of *C. difficile* were detected in both genders suffering from HA-D, in which 7.54 % of males and 6.3 % of females had positive PCR results. Similar results were also recorded among the genders who suffered from CA-D but with a lower percentage, in which 2.04 % of males and 1.9% of females were positive. Watery diarrhea was found in 51% of HA-D cases, all of whom were male who received both vancomycin and metronidazole, while bloody diarrhea was found in 47% of HA-D cases, all of whom were female. For CA-D, the diarrhea was watery for both genders and both amoxiclav and vancomycin drugs were used for treatment as shown in Table 4. No significant association was found between *C. difficile* infection and gender ($p < 0.97$).

Table 3. Results of PCR for the HA-D age groups.

Age groups	Number	PCR positive No. (%)
< 6 months	11	1 (9.1)
1-2 years	20	2 (10.0)
2-3 years	18	1 (5.5)
3-4 years	17	1 (5.8%)
4-5 years	19	2 (10.5)
5-6 years	15	0 (0.0)

HA-D: Hospital acquired diarrhea; PCR: Polymerase chain reaction.

Discussion

This cross-sectional study was performed to show the prevalence of *C. difficile* infection among both HA-D and CA-D cases using the conventional cultural method, enzymatic detection, toxin detection, and PCR. The study also investigated the association of risk factors with the antibiotics used for treatment and the development of infection by this pathogen. The results of this study clearly indicated that cultural methods using selective medium Cycloserine-Cefoxitin-Fructose agar (CCFA) (Condalab, Madrid, Spain) with the supplement (SIGMA-Aldrich, Massachusetts, USA) and 7 % horse blood were inefficient for isolating this pathogen from the stool samples, and other spore-forming Gram-positive clostridial species can grow on it in spite of using alcoholic shock which kills the vegetative form of bacteria allowing spore-forming bacteria to survive. The results of this study were dissimilar from others that found 20% and 28.6% of the stool samples from Iranian diarrheic patients to be

Table 4. Results of PCR according to gender, duration of hospitalization, antibiotic used, and type of diarrhea.

	Gender	Number	Duration of hospitalization in days	Antibiotic used	Type of diarrhea	PCR positive No. (%)
HA-D	male	53	2	vancomycin and metronidazole	watery	4 (7.54)
	female	47	2	penicillin, clindamycin, ampicillin	bloody	3 (6.3)
CA-D	male	49	3	amoxyclav	watery	1 (2.04)
	female	51	2	vancomycin	watery	1 (1.9)

PCR: Polymerase chain reaction; HA-D: Hospital acquired diarrhea; CA-D: Community acquired diarrhea.

positive by culture [22,23]. In Iraq, 8.1% and 21.25% of diarrheic patients were positive when tested by culture [24]. This discrepancy in the results can be attributed to many factors like methodology, identification tools, geographical location, seasonal variation, sanitary status of the hospitals and different ages. Several studies depend only on the phenotypic identification of this pathogen, which is not reliable unless confirmed by molecular techniques, because genus *Clostridium* includes more than 100 species which may share some phenotypic traits. Alcoholic shock using prior culturing destroys all vegetative forms of bacteria which may kill the vegetative form of *C. difficile* before transferring into spore and the presence of other species of *Clostridium* in high concentration may dominate the growth of low number of *C. difficile* or may be due to the stress caused by antibiotics on this pathogen. Out of 100 stool samples from HA-D cases, 19 (19%) samples showed growth of colonies which were spore-forming Gram-positive bacilli, but all of them were non-*C. difficile* as determined by PCR. Therefore, all growth on culture media should be confirmed by the PCR technique. Indeed, in recently published guidelines, nucleic acid amplification tools (NAATs), like the PCR technique, have been finally recognized as a superior method for diagnosing *C. difficile* [23]. Out of a total of 300 stool samples, 9 (3%) were positive for *C. difficile* by the PCR technique, in which 7 samples (7 %) among 100 stool samples were from HA-D cases and 2 (2%) from CA-D cases, which is similar to a previous study [22] that found that 10.3 % of Iraqi diarrheic patients were positive for *C. difficile* when tested with PCR and another [24] found that the rate of *C. difficile* infection ranged from 3-30 %. No *C. difficile* was detected among the control group by both the isolation and PCR techniques, which indicated a very low level of carriers among children in the area. This low level of carriers among children of area is due to the improvement in the socioeconomical level, hygienic status and increasing public health awareness. A high percentage (12.0%) of GDH positive samples was found among HA-D compared to a low percentage (2.0 %) among CA-D cases. These results were different from a previous study [5] that found that 23.8 % of the samples were positive for GDH. This difference in the results can be attributed to the different methodology and geographical location. Out of a total of 12 GDH positive stool samples identified by the immunochromatography strip method, only 7 (58.3 %) samples were identified as positive PCR, while another 5 samples were PCR negative; thus, indicating that this test method is not specific and gives false positive results. Toxin A and B detection by the immunochromatography strip method showed that 7 out of a total of 8 (87.5 %) positive samples for both toxins among HA-D cases were positive by PCR and two out of a total of 5 (66.6 %) samples were positive for both toxins among CA-D cases by the PCR method, and these results also indicated the presence of false positive results. No significant differences were found between *C. difficile* infection and increasing ages as well as genders. The most commonly associated antibiotic with the development of *C. difficile* infection was vancomycin.

Conclusions

Based on the results of this study, it can be concluded that isolation, GHD test and toxin test by immunochromatography strip method are unreliable for diagnosing *C. difficle* in stool samples. The PCR technique is more accurate for final diagnosis of this pathogen.

Authors' contributions

Study concept and design: Saeed, Abdulmawjood; sample collection: Ali; analysis and interpretation: Ali, Saeed, Abdulmawjood; drafting of the manuscript: Ali, Saeed; critical revision of the manuscript for important intellectual content: Saeed, Abdulmawjood; statistical analysis: Saeed; study supervision: Saeed, Abdulmawjood.

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Conflict of interests: No conflict of interests is declared.