J Med Microbiol Infec Dis, 2014, 2 (3): 95-99

Original Article

Detection of Virulence Genes of *Clostridium difficile* in Children with Cancer by Multiplex PCR

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Received May 27, 2015; accepted Sep 06, 2015

Introduction: Toxigenic *Clostridium difficile* is the major cause of antibiotic-associated diarrhea, colitis, and pseudomembranous colitis. The pathogenicity of *C. difficile* is related to toxins A&B. Children with cancer are at risk of developing *C. difficile* infection (CDI) due to increased exposure to antibiotics, immunosuppression, and longer hospital stays. Recently, due to higher sensitivity and specificity of nucleic acid amplification test (NAATs) compared to toxin enzyme immunoassays (EIAs), many laboratories are transitioning to NAATs for detection of CDI. We aimed to use a multiplex PCR to detect the *C. difficile* toxin genes *tcdA*, *tcdB* and *tpi* in stool of cancerous children. We also aimed to show the effects of chemotherapy regimens on the prevalence of *C. difficile* in these children. **Methods:** 105 fecal samples were collected from cancerous children who were hospitalized and undergoing chemotherapy. The presence of *tcdA, tcdB*, and *tpi* genes were tested. **Results:** *C. difficile* was identified in 17.14% of children and the detection rate of A^-B^+ strains was higher than A^+B^+ and A^+B^- strains. *C. difficile* was found in 17.8% of children who received single antibiotic (5/28 cases; virulence genes were detected in 4 cases) and in 41.4% of patients who received more than one antibiotics (12/29 cases; virulence genes were detected in 8 cases). **Conclusion:** Multiplex PCR is a powerful technique for preliminary screening and rapid detection of *C. difficile* and its virulence genes in the stool of cancerous children. The prevalence of *C. difficile* in cases receiving several antibiotics was higher than those receiving single antibiotics. *J Med Microbiol Infec Dis, 2014, 2 (3): 95-99.*

Keywords: Antibiotic Prophylaxis, Cancer, Toxigenic, *Clostridium difficile*, Multiplex PCR.

INTRODUCTION

Clostridium difficile is an anaerobic, spore forming, and gram-positive bacteria, which is the cause of gastroenteritis and pseudomembranous colitis and the main cause of diarrhea in hospitalized patients. *C. difficile* is commonly isolated from feces of children and adults [1]. Hall and Toole identified *C. difficile* as a normal component of infant intestine flora [2]. *C. difficile* produces toxin A (enterotoxin) and toxin B (cytotoxin) [3-7]. Toxigenic strains of *C. difficile* are responsible for 10-25% of antibiotic-associated diarrhea, leading to pseudomembranous colitis [8]. These toxins are encoded by *tcdA* and *tcdB* genes, which are located in a pathogenicity locus within the chromosome of *C. difficile* [9] and produced after intestinal colonization, leading to injury to the intestinal mucosa [6]. Although, most pathogenic strains of *C. difficile* are A^+B^+, some pathogenic A^-B^+ strains have been reported [10]. A^-B^+ Strains have a deletion in the region of second repetition of Toxin A gene, which encodes an epitope that interacts with toxin A antisera [9]. The role of A^+B^- isolates in infection remains unclear, but it has been suggested that their toxin may result in a different disease manifestation [11]. The frequent administration of antibacterial chemotherapy in such patients, gastrointestinal toxicity of anti-neoplastic chemotherapy, and possibly environmental exposure to the microorganism are the factors leading to *C. difficile* infection (CDI) in an individual patient [12, 13]. Children with cancer account for a large proportion of pediatric CDI cases.

Antibiotics therapy (especially broad-spectrum antibiotics) and chemotherapy are the major risk factors of *C. difficile* infection [4]. The most commonly used antibiotics are broad-spectrum antibiotics (penicillins, cephalosporins and clindamycin), which have significant adverse effects on normal intestine flora [14]. Long-term combined antibiotics therapy increases the risk of *C. difficile* colonization and *C. difficile*-associated diseases (CDAD) [15, 16]. In cancerous patients under chemotherapy, the normal intestinal flora is disrupted, leading to *C. difficile* (especially toxigenic strains) growth and intestinal infection. Traditionally, *C. difficile* toxins are identified by ELISA, EIA, and culture on CCFA medium.

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These methods are accurate and sensitive, but are time consuming and have low sensitivity. Although, toxin B is detected by its cytotoxic activity on cell culture, but this method is not used in clinical microbiology laboratories [17].

Molecular methods, such as PCR, RT-PCR and multiplex PCR can be used as accurate and sensitive methods in the identification of toxins [18]. In this study, the presence of *tcdA*, *tcdB*, and *tpi* genes in stool samples of cancerous children was assessed by multiplex PCR technique. We also aimed to show the effect of chemotherapy and antibiotic therapy on the prevalence of *C. difficile* in children with cancer.

MATERIAL AND METHODS

Patients and fecal specimens. In a cross-sectional study, between May 2011 to March 2012, stool specimens (n=105) were collected from children (<15 years of age) with cancer who were hospitalized and undergoing chemotherapy. Stool specimens were collected from oncology unit of Mahak, Imam Hussein hospital, and Children' Medical Center in Tehran, Iran. Calculation of sample size was performed according to the average prevalence of 10-25% of toxigenic *C. difficile* with 95% confidence level and error of 0/04. Sampling was performed according to the procedure for stool collection from children under 15 years with cancer who were undergoing chemotherapy. Information on some clinical and epidemiological features was obtained through questionnaires. The patients were pretreated with drugs and antibiotics. The specimens were obtained by sterile swabs, placed in sterile tubes, kept at 4˚C, and analyzed within 24 to 36 h of collection.

Sample handling and DNA extraction. Fecal swabs were placed in 1.0 ml of TE buffer or DDW and vortexed several times. Then, the tube content was transferred to Eppendorf tubes and centrifuged at 1000-1500 rpm for 1-2 min. Supernatants were decanted to new tubes and centrifuged at 14000 rpm for 3 min. About 400 µl of supernatant was thrown away and the remaining was vortexed and stored at -20°C. A QIAamp DNA stool mini kit (Qiagen, Germany) was used to extract total DNA from stool samples, and 10 µl of it was used as template DNA for PCR.

Multiplex PCR for identification of *C. difficile* and its virulence genes. As an internal control, all stool-extracted DNAs were checked by universal primers, which amplified a conserved region of 23S rDNA. The stool-extracted DNA samples that produced no amplicons using the universal primers, were omitted from the study. To detect the selected genes from *C. difficile*, multiplex PCRs were done on the prepared DNA using reported primers [19]. The reaction was performed in an Eppendorf Mastercycler in a final volume of 35 µl containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 1 µM of each primer [except for *tpi-F* and *tpi-R* (0.5 µM)], 4 units of *Taq* DNA polymerase, and 10 µl of extracted DNA sample.

The PCR mixtures were denatured (3 min at 95°C), and then a touchdown procedure was implemented, consisting of 30 s at 95°C, annealing for 30 s at temperatures decreasing from 65 to 55°C during the first 11 cycles (with 1°C decremental steps in cycles 1 to 11), and a final extension step at 72°C for 30 s. A total of 40 cycles were performed. PCR products were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. In positive samples, the multiplex PCR products were eluted from the gel and sequenced by GATC Company (Germany).

Analysis of primers' sensitivity. Sensitivity of the primers was assessed in aliquots of 0.1 ml of serial dilutions from 10^9 to 10^2 bacteria/ml, obtained from an overnight growth of *C. difficile* toxigenic strain ATCC 9689, which were transferred into 0.9 ml of *C. difficile*-negative liquid stool. Concentrations of inoculated stools (range, 10^8 to 10 bacteria/g of stool) were obtained and tested by PCR assay. Respective stool pellets obtained through the processing were tested using the PCR assay.

RESULTS

The sensitivity of the PCR assay was determined by spiking fecal samples with different concentrations of toxigenic strains of *C. difficile*. The detection limit was about 5×10^4 CFU/g of feces, and the analytic sensitivity of the technique was calculated to be 25 pg DNA per PCR reaction. To evaluate the applicability of the assay to detect *C. difficile* in stool of 105 cancer patients under chemotherapy, fecal samples collected from hospitalized patients were tested. All fecal samples were either unformed or of liquid consistency, as recommended for *C. difficile* testing. *C. difficile* was detected in stools of 18 of 105 (17.14%) cases, aged between 2 months and 15 years (Table 1 summarizes their demographic characteristics). All PCR products of *tpi* gene were sequenced and showed high specificity of the test. The amplification control of the PCR showed no significant inhibition by any of the samples tested.

A total of 18 (17.14%) samples from cancerous children were positive for *C. difficile* strains, of which 13 (72%) cases were toxigenic. In toxigenic samples, 4 cases were A^+B^+, 1 case was A^+B^-, and 8 cases were A^-B^+ (Figure 1).

Fifty-seven children received antibiotics during the sampling period, of whom 28 (26.6%) received a single antibiotic and 29 (27.6%) received several antibiotics, but the remaining 48 (45.7%) children received no antibiotics. Among those using a single antibiotic, 5 (17.8%) patients had *C. difficile* in their stools, of which 4 (80%) were toxigenic genes-positive. *C. difficile* was found in the stool of 12 (41.4%) patients who used more than one antibiotic, of which 8 (66.6%) strains were toxigenic genes-positive (Table 2).

In cases treated with single antibiotics, the prevalence of *C. difficile* was higher in those who received third generation cephalosporin (ceftriaxone, ceftazidime, ceftazidime, and cefazolin), compared to those who received other antibiotics. The prevalence of *C. difficile* in cases receiving several antibiotics was higher than those receiving single antibiotics ($p\leq0.05$).

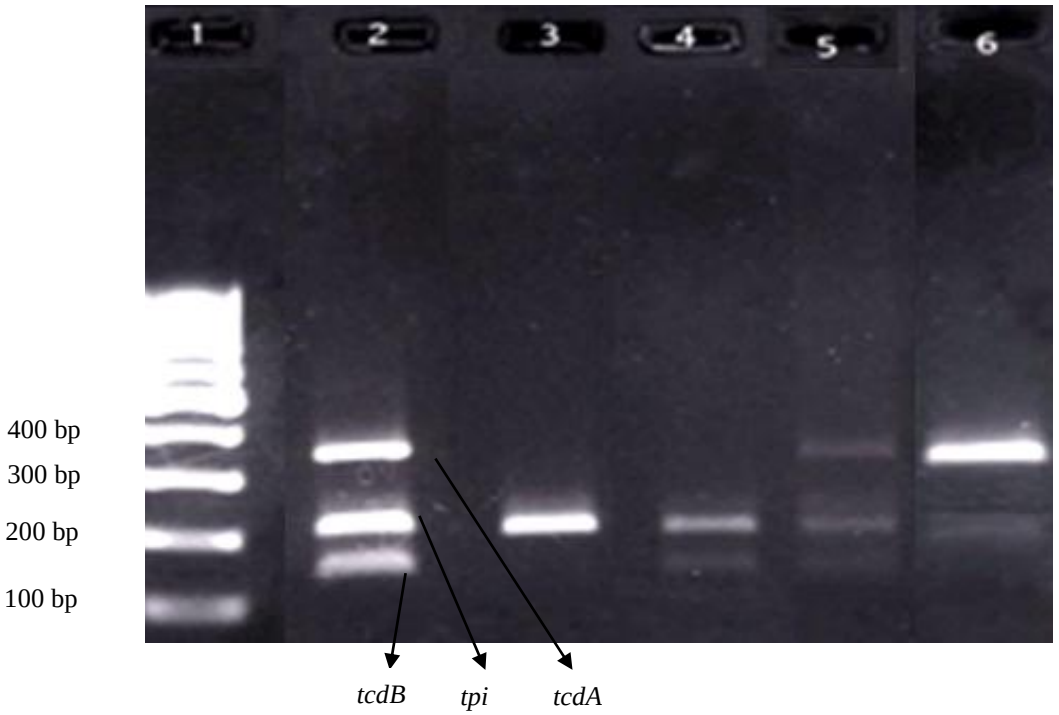

Fig. 1. Multiplex PCR of the reference strain and clinical strains. Lane 1, molecular weight standard 100 bp (Fermentase); lane 2, *C. difficile* ATTC 9689; lane3, non-toxigenic (A^-B^-) *C. difficile* strain; lane 4, A^-B^+ *C. difficile*; lane5, toxigenic (A^+B^+) *C. difficile* strain; and lane 6, A^+B^- *C. difficile* strain.

Table 1. Demographic characteristics and chemotherapy regimen of cancerous children carrying *C. difficile*

***C. difficile* positive (n:18)**	**age**	**sex**	**Underlying disease**	**Chemotherapy duration**	**Antibiotics**
1	11 years	Male	Burkitt Lymphoma	18 month	Vancomycin, Meropenem, Ticoplatin, Metronidazole
2	6 years	Male	PNET	24 month	Vancomycin, Meropnem
3	5 years	Female	Major Thalassemia	24 month	Vancomycin, Meropenem, Metronidazole, Cotrimoxazole
4	11 years	Female	ALL	6 month	Meropenem, Ceftriaxone
5	3 years	Female	Retinoblastoma	17 month	Ceftazidime
6	4 years	Male	Ependymoma	3 month	Vancomycin, Meropnem
7	3 years	Female	Rhabdomyosarcoma	18 month	Metronidazole, Imipenem
8	5 years	Female	AML	20 month	Cefazolin
9	2 years	Female	ALL	9 month	Metronidazole, Amikacin
10	9 month	Male	Neuroblastoma	6 month	Cefazolin, Amphotericin
11	9 years	Female	Retinoblastoma	6 month	Ceftazidime
12	2 years	Female	ALL	2 month	Ceftazidime Vancomycin
13	1 year	Female	ALL	13 day	Ceftriaxone
14	7 years	Female	Rhabdomyosarcoma	2 month	Ceftazidime, Meropenem
15	6 years	Male	ALL	6 month	Ceftazidime Vancomycin, Clindamycin
16	12 years	Male	Rhabdomyosarcoma	11 month	Ceftizoxime
17	7 years	Male	ALL	12 month	Methotrexate
18	2 years	Male	NHL	8 month	Imipenem, Vancomycin

Table 2. Antibiotic treatment in children with toxin genes-positive *C. difficile*

	Patients	**Positive cases**	**Negative cases**
Single antibiotic	28	5	23
Combined antibiotic	29	12	17

DISCUSSION

Children are increasingly being recognized as an emerging population at risk for CDI. Children with cancer have an increased risk of developing CDI due to increased antibiotic exposure, immunosuppression, and longer hospital stays. Nowadays, patients with cancer receive more intensive chemotherapeutic regimens together with broad-spectrum antibiotics during periods of intense immunosuppression. Thus, cancer patients are susceptible to colonization with *C. difficile*, but the role of this pathogen in pediatric oncology patients is poorly understood [20]. In this study, we determined the prevalence of toxigenic *C. difficile* strains in cancerous children by multiplex PCR. We detected *tcdA*, *tcdB*, and *tpi* genes in stool specimens using 3-plex PCR. Persson *et al.* studied *tcdA*, *tcdB*, *cdtA*, and 16S rDNA genes by 5-plex PCR [21].

C. difficile was detected in 17.3% of pediatric cancer patients receiving chemotherapy, and in 72% of these cases, toxigenic genes were detected. These results were unlike those of Burgner *et al.*, who investigated colonization of *C. difficile* in cancerous children, and concluded that *C. difficile* does not appear to be an important pathogen in children and may be considered as a part of the normal flora [22].

Bacteriological culture is the gold standard and the most accurate method for the identification of *C. difficile*, but it is time-consuming and not very specific for isolation of non-toxigenic strains. EIA is a less sensitive test that should always be combined with culture [17]. Nowadays, toxin detection from stool specimens is a prerequisite for the diagnosis of CDI [5]. Molecular methods, such as PCR, real-time PCR, and multiplex PCR can be used as accurate and sensitive methods in identification of toxigenic genes. Molecular tests (DNA-based tests) are useful methods for diagnosis of CDI. At least four FDA-approved nucleic acid amplification assays are available to clinical laboratories, some of which have been well evaluated in the literature. Since these assays detect a gene that encodes toxin and not the toxin itself, it is important that laboratories test only patients with diarrhea. The BD GeneOhm Cdiff Assay, is a real-time PCR assay targeting the toxin B gene [23]. In this study, we used multiplex PCR method because this method is fast and can detect several genes simultaneously in one reaction. In addition, multiplex PCR can differentiate toxigenic from non-toxigenic strains [19].

Isolation of *C. difficile* is not sufficient for diagnosis, because more than 20% of isolates do not produce toxins and therefore are not pathogenic. Additional testing by tissue culture or EIA must be performed to show that the isolates can produce toxin [24].

It has been reported that with intensive chemotherapeutic protocols used in pediatric cancer patients, *C. difficile* colonization occurs with increased frequency. Current treatment of cancer patients is often performed by broad-spectrum antibiotics and antineoplastic drugs. These policies lead to increased susceptibility of these patients to CDI [24]. Bignardi reported that use of antibiotics increases the risk of CDI. Probably, antibiotic therapy is an important factor in the acquisition of *C. difficile* [14]. The results of this study are in agreement with those of other studies that showed the prevalence of *C. difficile* is higher in cases treated with combined antibiotics compared to those treated with single antibiotics [25].

In summary, rapid diagnosis of *C. difficile*-associated diseases is necessary to initiate a specific treatment and to take appropriate measures to control nosocomial spread, but monitoring pathogenic variant strains is also important for better evaluation of the relevance of the diagnostic tests in each hospital laboratory. Our developed multiplex PCR on DNA samples directly isolated from feces may be proposed as an improved diagnostic approach for identification of human and animal *C. difficile* intestinal infections, providing combined species identification and toxigenic type characterization.

Chemotherapy regimen in cancerous children could increase the probability of developing CDI. Rapid detection of toxigenic *C. difficile* in cancerous children could help in the prevention of CDI. Multiplex PCR provides information on the presence of *C. difficile* and its toxins within a few hours, in contrast to standard culture-dependent methods, in which detection results can be obtained only after 3 days or more (48 h for culture and 1 day for toxin detection). Direct PCR on DNA isolated from stool samples is convenient, rapid, and useful for the preliminary detection of toxigenic types of *C. difficile* in fecal samples.

ACKNOWLEDGEMENT

We would like to thank Dr. Anis jafari (Department of Molecular Biology, Pasteur Institute of Iran) for her great help and advice on this study.

This project was supported by the Pasteur Institute of Iran.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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