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

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**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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http://jommid.pasteur.ac.ir
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 QIAaamp 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 amplex non-amplifications 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 MgCl₂, 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 10⁰ to 10² 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, 10⁰ 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 5×10⁴ 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 (ceftiraxone, ceftazidime, cefazidime, 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≤0.05).
Fig. 1. Multiplex PCR of the reference strain and clinical strains. Lane 1, molecular weight standard 100 bp (Fermentase); lane 2, *C. difficile* ATCC 9689; lane 3, non-toxigenic (A-B-) *C. difficile* strain; lane 4, A-B+ *C. difficile*; lane 5, 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*

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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patients</th>
<th>Positive cases</th>
<th>Negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single antibiotic</td>
<td>28</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Combined antibiotic</td>
<td>29</td>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>
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 toxicogenic 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.

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CONFLICT OF INTEREST

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

REFERENCES


