**Clostridium difficile** infection in cancer patients with hospital acquired diarrhea at the teaching hospitals in Iran: Multilocus sequence typing analysis (MLST) and Antimicrobial resistance pattern*

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**Key words:** Clostridium difficile infection, antibiotics, molecular epidemiology, multilocus sequence typing, cancer patients

**Parole chiave:** Infezione da Clostridium difficile, antibiotici, epidemiologia molecolare, multilocus sequence typing, pazienti oncologici

**Abstract**

**Background.** This study aimed to investigate the phenotype and genotype characterization of Clostridium difficile isolates among cancer patients with hospital-acquired diarrhea in 4 teaching hospitals in Isfahan, Iran.

**Study design.** This was a cross sectional study conducted on adult (>18 years old) between April 2015 and May 2017.

**Methods.** Over two years, 67 diarrheic fecal samples were collected. C. difficile isolates were characterized according to the presence of toxin genes and antibiotic resistance. Multilocus sequence typing (MLST) was performed to evaluate the genetic relationships between different lineages of toxigenic strains.

**Results.** Seven toxigenic and 12 non-toxigenic strains were detected among stool samples. Patients with a history of previous surgery during hospitalization were more than 7 times likely to develop Clostridium difficile infection (CDI). All isolates were susceptible to metronidazole, vancomycin and fusidic acid. Toxigenic C. difficile strains were divided into 3 different sequence types. The detected types were ST-54, ST-2 and ST-37, while none of the isolates was identified as ST-1 or ST-11.

**Conclusions.** This is the first description of the MLST analysis of C. difficile strains isolated from cancer patients in Iran. All of the studied population were exposed to multiple antibiotics and chemotherapeutic agents. Further research and clinical studies are recommended in the treatment through good antimicrobial stewardship and prevention of C. difficile infection in all healthcare settings.
Introduction

*Clostridium difficile* is a spore forming, strictly anaerobic, gram-positive bacillus, identified as the prevalent cause of healthcare-associated infectious diarrhea (1, 2). *Clostridium difficile* Infection (CDI) is the frequent nosocomial infection after long-term antimicrobial treatment (3). The clinical features of this toxin producing, opportunistic pathogen can range from mild diarrhea to pseudomembranous colitis or toxic mega colon (2, 4). Cancer patients are at increased risk for CDI because of their underlying malignancy, prolonged antibiotic treatment or chemotherapy, radiation therapy, frequent or prolonged hospitalizations, and depressed immune response (1, 2, 4).

Previous studies related to CDI were based on epidemiologic data and incidence of toxigenic *C. difficile* obtained from hospital and non-hospital setting (3, 5). Diarrhea is a common adverse effect of chemotherapy and CDI can be unrecognized that leading towards serious morbidity and mortality in cancer patients (1, 6). The incidence of CDI among hospitalized cancer patients differs nationwide (1). The rate of CDIs was increased between 2000 and 2010, there were about 15 cases per 1,000 hospital discharges and 20 cases per 100,000 person-years in the community (6).

Previous studies have reported a nine fold and 1.4-fold higher incidence of CDI among hematopoietic stem cell transplant recipients and other cancer patients, respectively, compared to any other patient population (2, 6).

CDI has appeared as a challenge in healthcare settings because of an increased virulence, the emergence of more resistant strains to treatment, and an expanding at-risk population (2, 6, 7).

Epidemiologic characteristics and molecular typing of CDI cases are important tools for the investigation of hospital outbreaks and understanding the modes and transmission sites (1, 8).

Considering the absences of information and diagnosis of CDI in cancer patients in our region, this study aimed to investigate the phenotypical and molecular characterization of toxigenic and non-toxigenic *C. difficile* isolates from the unformed stool of hospitalized patients developing diarrhea in oncology wards of teaching hospitals in Isfahan, Iran.

Materials and Methods

Study design

This was a cross sectional study conducted on adult (>18 years old) cancer patients with presence of hospital-acquired diarrhea at major teaching hospitals in Isfahan, the central part of Iran between April 2015 and May 2017. The demographic information of patients such as age, gender and their independent risk factors i.e. previous surgery, the drugs used by patients and antibiotic treatment within 2 months before the time of *C. difficile* detection were recorded. Diarrhea was determined as the passage of loose stool three or more times per day which starts three or more days after hospitalization (9).

Stools specimens were screened for the presence of other enteropathogenic organisms (*E. coli*, *Salmonella* specious, *Shigella*, and *Campylobacter jejuni*).

*Clostridium difficile* culture

Stool samples were examined by inoculation into a selective enrichment broth (*C. difficile* moxalactam norfloxacin broth [CDMNB] supplemented with cysteine hydrochloride, norfloxacin, moxalactam, and 0.1% sodium taurocholate (Oxoid, UK) in an anaerobic jar for 5–7 days. Preliminary treatment with alcohol shock was performed in order to recover *C. difficile* from stool specimens. The pellets were then inoculated by a sterile loop onto the CDMN- agar surface supplemented with 7% sheep blood and incubated anaerobically for 48 h at 37°C.
Suspect colonies with 2-3 mm in diameter, p-cresol odor, ultraviolet fluorescence (365 nm), Gram stain morphology, and biochemical reactions such as L-proline aminopeptidase test (Prodisk, Remeb, Lenexa, KS, USA) were identified as C. difficile strains (5, 10, 11). These colonies were inoculated onto the Brucella agar with 5% defibrinated sheep red blood cells for molecular tests and were confirmed by testing with tpi gene PCR amplification. (1, 5, 10). C. difficile ribotype 027 was used as positive control for molecular and microbiological analysis. C. perfringens 450 MTCC (Microbial Type Culture Collection) was used as the negative control (5). DNA extraction was performed using the modified Pitcher et al., procedure (1989). Briefly, 10 ml of Brain Heart Infusions (BHIs) was inoculated with a colony of C. difficile and was incubated overnight in a 37°C anaerobic chamber. Cells were harvested by centrifuging, the cell pellet was washed in TE buffer (Tris, 10 mM; EDTA, 50 mM; pH 8.0), resuspended in TE buffer and lysozyme solution (50 mg ml⁻¹). Guanidium thiocyanate and sarkosyl were added to the mixture for protein denaturation. (12, 13). DNA was purified by chloroform-isoamyl alcohol. The precipitate was washed with 70% ethanol, dehydrated and dissolved in sterile deionized water and stored in a -20°C freezer until use (14). All isolates were screened for the presence of the genes encoding toxin A and B (tcd A and tcd B), binary toxin (cdt B) and triose phosphate isomerase (tpi) were amplified by PCR using known primers and condition as described by Stubbs et al and Lemee et al., (5, 10, 15).

Multilocus sequence typing (MLST)

MLST was carried out and analyzed for C. difficile strains according to the previous articles (9). MLST was performed with seven housekeeping genes (adk, atpA, dxr, glyA, recA, sodA, and tpi) on detected toxigenic isolates as described previously by Griffiths et al., (16, 17). The information for C. difficile alleles and sequence types (STs) were established from C. difficile MLST database, which is approachable at http://pubmlst.org/clostridium_difficile. The amplified products were sent to Bioneer Corporation in South Korea for sequencing. The DNA sequences of the 7 genes were submitted to the MLST database to obtain the sequence type (ST).

Antibiotic susceptibility tests

E-tests (Liofilchem®, Italy) determined Minimum inhibitory concentrations (MICs) of vancomycin (VAN), metronidazole (MTZ), moxifloxacin (MXF), fusidic acid (FU), rifampin (RIF) and clindamycin (CLI) to be used according to the manufacturer’s instructions. All tests performed on Brucella Blood Agar plates containing vitamin K1 (1 mg/ml), haemin (5 mg/L) and 5% defibrinated sheep red blood cells. The procedure and interpretation of results were carried out according to the Clinical and Laboratory Standards; the breakpoints used were 8 μg/ml for Clindamycin, 4 μg/ml for moxifloxacin; 2 μg/ml for vancomycin and 32 μg/ml metronidazole; rifampin; fusidic acid as described previously (18, 19).

Statistical Analysis

Data were presented as the mean ± standard deviation. All probabilities were two-tailed and a P value of < 0.05 was defined as statistically significant.

The logistic regression model was used to determine the clinical factors associated with C. difficile colonization. First, a univariate logistic regression model was fitted on each independent variable, and then a multivariate regression model with adjustment for the effects of other covariates was used. Variables that were significant in univariate models were entered into the multivariate model. Selection of variables in the multivariate model was based on a stepwise procedure. We estimated Odds Ratios (ORs) and 95% confidence intervals for each of clinical
factors using logistic regression models. Statistical analysis was performed using the statistical software SPSS, version 16.

Results

Faecal samples from 67 cancer patients were studied over two years period (April 2015 - May 2017). Of the 67 patients, 19 (28.3%) were *C. difficile* culture positive at screening including seven or 10.4% with CDI and 60 with non-CDI (Table 1). The median age of patients with CDI was 53.8 years old (range 39 -71) and the frequent oncologic diagnosis of the patients with CDI was breast cancer patients at 42.8% (3/7) rate. The use of antibiotic was identified in all patients. Clindamycin, ciprofloxacin, and metronidazole were the most frequently used antimicrobial agents in those patients. Most patients were using immunosuppressive therapies, chemotherapeutic agents, and antibiotics. These patients were treated with one to three antibiotics and approximately 40% of CDI patients had used two or three antibiotics (Table1).

The stepwise multivariate logistic regression model revealed that patients with chronic renal diseases were at six fold increased risk for acquiring non-toxigenic *C.difficile* strains (OR 6; 95% CI, 1.116-32.24; P= 0.002). This study found an association between the gastroenteritis and lower

Table 1 - Clinical characterizations of 67 Cancer patients with diarrhea in CDI and Non CDI groups admitted to the teaching hospitals, Isfahan, Iran (2015-2017)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CDI Patients, n = 7</th>
<th>Non CDI Patients, n = 60</th>
<th>Non-Toxigenic strains, n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxigenic <em>C. difficile</em> Strains (A+/B+ A-B+)</td>
<td>Negative <em>C. difficile</em> strains, n = 48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Median age, years</td>
<td>53.8 ± 13.7</td>
<td>52.6 ± 14.7</td>
<td>45.9 ± 14.8</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>3 (42.8)</td>
<td>28 (58.3)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>A number of antibiotics used in previous 8 weeks, (cephalosporin, aminoglycoside, clindamycin, metronidazole, …)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>1(14.3)</td>
<td>7 (14.6)</td>
<td>0</td>
</tr>
<tr>
<td>Two</td>
<td>3 (42.8)</td>
<td>24 (50.0)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Three</td>
<td>3 (42.8)</td>
<td>17 (35.4)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Chronic renal diseases</td>
<td>2 (28.6)</td>
<td>4 (8.3)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Gastroenteritis diseases</td>
<td>4 (57.1)</td>
<td>8 (16.7)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Enteral tube feeding</td>
<td>5 (71.4)</td>
<td>31 (64.6)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>Receipt antacid medications</td>
<td>5 (71.4)</td>
<td>32 (66.7)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Previous surgery</td>
<td>6 (85.7)</td>
<td>23 (47.9)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>7 (100)</td>
<td>43 (89.6)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>Chemistry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xeloda® (capecitabine)</td>
<td>3 (42.8)</td>
<td>12 (25.0)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>5FU or Taxol®</td>
<td>4 (57.2)</td>
<td>23 (43.7)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>5 (10.4)</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

Legend: 5FU 5-fluorouracil, Taxol® paclitaxel
likelihood of acquiring CDI when compared to those with non-toxigenic *C. difficile* strains (OR 0.08; 95% CI, 0.008-0.77; P = 0.03). The study findings also revealed that patients with a history of previous surgery during hospitalization were more than seven times more likely to develop CDI (OR 7.36; 95% CI, 1.34-40.5; P = 0.02) (Table 2).

None of the specimens were positive for toxin A alone or binary toxin. We identified three (3) different sequence types among toxigenic strains (ST 54, ST 2 and ST 37). The most common ST was 54 (*tcdA*+, *tcdB*+, *cdtB*-) accounting for 57.1% (4/7) of all strains (Table 3).

All t strains were susceptible to metronidazole, vancomycin, and rifampin. All the toxigenic isolates were inhibited by a low concentration of vancomycin (MIC < 0.5 μg ml⁻¹). Approximately 43% (3/7) and 28.6% (2/7) of toxigenic isolates were resistant to clindamycin and moxifloxacin, respectively (P>0.05).

**Discussion and Conclusion**

In this study, we demonstrated a relatively high rate of *C. difficile* colonization with toxigenic and non-toxigenic strains (23.8%,...
19/67) in cancer patients with diarrhea in four major teaching hospitals in central Iran. Due to unavailability of *C. difficile* culture and toxin testing (procedure or kits) in many hospitals of Iran, awareness of circulating strains and their prevalence has been limited. The incidence of CDI in our patients was estimated 10.4%, which is lower than those reported in the cancer hospitals in Spain (17.3%), Brazil (48%) and China (23.4%) (1, 2, 6). Four isolates were positive for both toxin A, toxin B and three isolates for toxin B. However, none of the seven isolates carried binary toxin genes, the presence of which is related with higher mortality and recurrence rates (15, 20). Our strains exhibited low genetic diversity and were genotyped into 3-STs, not specific to Iran (ST 54, ST 2 and ST 37); a finding which suggests the worldwide spread of some lineages. No correlation was found between MLST and toxin phenotypes. Previous reports from China showed various MLST genotypes, which ST-54, ST-2, and ST-37 were the most common types (20). The *C. difficile* strain BI/NAP1/027(ST-1) was not found in the current research, which is responsible for the high rate of recurrence and mortality. Other recent studies in China have been reported this strain as a prevalent cause of CDI (20, 21). This might be due to the different geographical locations where the study has been carried out.

The strains isolated in this study showed sensitivity to metronidazole, fusidic acid, and vancomycin. Resistance to vancomycin has not been observed frequently in *C. difficile*; although some strains with reduced susceptibility to vancomycin have been reported in Iran and China. Therefore, this antibiotic is found to be effective for the treatment of patients with moderate to severe CDI (20, 22). In this study, 43% (3/7) of toxigenic isolates were discovered to be resistant to clindamycin and 28.6% (2/7) to moxifloxacin. Most studies have exhibited that certain antibiotics such as clindamycin and fluoroquinolones carry a higher risk for CDI than others cite a reference. Resistance was largely observed towards clindamycin (82%), ciprofloxacin (98%) and Clindamycin (82%) (23). Low susceptibility to these antimicrobial agents has been reported in other studies that can be attributed to different antibiotic regimens used and the environmental factors (20, 22, 23). There was no significant difference in the resistance rates between toxigenic and non-toxigenic strains with respect to their susceptibility to these antibiotics.

Despite a possible decrease in the incidence of CDI in current decades, the *C. difficile* colonization/infection of cancer patients contributes significantly to morbidity and possibly mortality significantly. The reason may be due to diarrhea which often results in reducing or delaying chemotherapy or radiotherapy (1, 2, 24).

Different antineoplastic drugs, antimicrobial agents, antacid medication, and corticosteroids were used in the treatment of all patients in this study. The study found no differences in drug treatments between CDI and non-CDI patients. The studies found no association between proton pump inhibitor therapy and severe CDI, which attributed this to the acid resistance of *C. difficile* spores (2, 9). Based on our multivariate regression model results, the patients who had a history of surgery 30 days prior were 7 times more likely to develop CDI. Extended antibiotic use in the postoperative period, longer preoperative length of hospital stay and the high rate of preoperative prophylactic antibiotics use have been reported as a strong predictor of CDI since the risk of colonization of *C. difficile* will be increased (25, 26).

Our results revealed that patients with chronic renal diseases were at a 6 fold increased risk for non-toxigenic CdC. The advanced chronic renal disease is a strong risk factor for CDI. Intestinal dysmotility leads to gastric acid suppression or microorganism overgrowth in these patients which may contribute to increased *C. difficile* colonization/
infection (27). We found an association between the gastroenteritis diseases and lower likelihood of acquiring CDI compared those with acquiring non-toxigenic C. difficile strains. The lower CDI incidence in patients with gastrointestinal diseases may be due to better or special care of the digestive tract in these patients (2, 11).

There are several limitations to our study including the sample size, which might represent a potential limitation and the cross sectional nature of the data does not permit to obtain any cause-effect relationship between drugs and CDI. CDI was diagnosed with stool culture and PCR for all specimens. However, stool cultures may result in low rates of toxin producing and non-toxigenic strains under these conditions. Therefore stool direct real time with additional verification is required which offers superior sensitivity and lower complexity limits.

This study provides information about different aspects of molecular epidemiology, clinical characteristics, and antibiotic resistance profiles of circulating C. difficile strains among Iranian cancer patients with hospital acquired diarrhea in large teaching hospitals during 2015 and 2017. This is the first description of MLST analysis of C. difficile strains isolated from cancer patients in Iran. ST-54, ST-2, and ST-37 were diagnosed as three types of strains that were also among the most common STs in the MLST database. The study population was exposed to multiple antibiotics and chemotherapeutic agents. Further research and clinical studies are recommended in the treatment through good antimicrobial stewardship and prevention of C. difficile infection in all healthcare settings. In addition, more studies with a larger panel of clinical samples should be performed to evaluate the epidemiology of C. difficile in this high-risk group.

Acknowledgement: This study was approved by the human research ethics committee of Isfahan University of Medical Sciences (the grant No. 291235), and it was carried out in accordance with the approved guidelines. We would like to thank Mr. Abbas Daei naser and Mrs. Rezvan Shafiei for their technical assistance. We would like to thank Prof Hamid Jaffari for his valuable comments.

Riassunto
Infezione ospedaliera da Clostridium difficile in pazienti oncologici con diarrea in Iran: tipizzazione tramite la tecnica di multi locus sequence typing (MLST) e profilo di antibiotico resistenza

Obiettivi. Lo studio ha indagato la caratterizzazione fenotipica e genotipica dei ceppi di Clostridium difficile isolati dai pazienti oncologici con diarrea acquisita in ospedale in 4 ospedali universitari ad Isfahan, Iran.


Results. Sette ceppi tossigenici e 12 non-tossigenici sono stati individuati tra i campioni di feci. I pazienti con una precedente storia clinica di chirurgia durante l’ospedalizzazione presentavano una probabilità 7 volte maggiore di sviluppare infezione da Clostridium difficile (CDI). Tutti gli isolamenti erano sensibili a metronidazolo, vancomicina e acido fusidico. I ceppi tossigenici di C. difficile erano suddivisi in 3 differenti gruppi. Quelli individuati sono stati ST-54, ST-2 e ST-37, mentre nessuno appartenne ai gruppi ST-1 o ST-11.

Conclusioni. Questa è la prima descrizione di una tipizzazione tramite la tecnica di multi locus sequence typing (MLST) di ceppi di C. difficile isolati da pazienti oncologici in Iran. Tutte le popolazioni incluse nello studio erano esposte ad agenti antibiotici e chemioterapici multipli. Ulteriori ricerche e studi clinic sono raccomandati per il miglioramento nella gestione dell’antibiotico resistenza e nella prevenzione delle infezioni da C. difficile negli ospedali.

References


