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Isolation of *Clostridium difficile* and Detection of A and B Toxins Encoding Genes

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Background: *Clostridium difficile* is the most important anaerobic, gram positive, spore forming bacillus which is known as a prevalent factor leading to antibiotic associated diarrheas and is the causative agent of pseudomembrane colitis. The role of this bacterium along with the over use of antibiotics have been proved to result in colitis. The major virulence factors of these bacteria are the A and B toxins.
Objectives: The purpose of this study was to isolate *C. difficile* from stool samples and detect A and B toxins encoding genes, in order to serve as a routine method for clinical diagnosis.
Materials and Methods: Recognition of A and B toxins encoding genes by uniplex and multiplex PCR using two pairs of primers from 136 accumulated stool samples.
Results: Results of the present study showed that out of 136 stool samples, three *C. difficile* were isolated and these strains contained A and B toxins encoding genes.
Conclusions: It was concluded that although detection of *C. difficile* from stool samples based on PCR (polymerase chain reaction) is expensive, yet this method is more sensitive and less time-consuming than culture methods and can be used as a clinical laboratory test.

Keywords: Clostridium difficile; Enterocolitis, Pseudomembranous; Polymerase Chain Reaction

1. Background

Clostridium difficile (CD) is a major cause of antibiotic-associated diarrhea (ADD) and the frequency of *C. difficile* infection (CDI) has significantly increased during the recent years (1, 2). It is responsible for approximately 15-25% of cases of antibiotic-associated diarrhea (AAD) and more than 95% of cases of pseudomembranous colitis (3-5). Infection with these bacteria is known as the leading cause of nosocomially acquired diarrhea in adults and can be responsible for large outbreaks (6). The new hyper virulent type (ribotype 027, pulse-field NAP1, toxinoty pe III) in several European countries and North America has been associated with more severe and fatal cases (7). For example, in Canada an increase in *Clostridium difficile* associated disease (CDAD) from 35.6 cases per 100,000 individuals in 1991 to 156.3 per 100,000 in 2003 was reported and in the United Kingdom (UK) a six fold increase in *Clostridium difficile* infection (CDI) related mortality was observed from 1999 to 2006 (8). Recently, cases of CDI caused by ribotype 027 strain have been reported in Asia. Few studies in Iran used culture for isolation of *C. difficile* from stool specimens.

Several phenotypic and molecular methods have been pragmatic to determine the relationship between strains of *C. difficile* (9). To effectively define *C. difficile* epidemiology, all sources of *C. difficile* need to be accurately identified, with organism recovery for molecular typing. This demands sensitive methods for of *C. difficile* detection. *C. difficile* generates two toxins, (toxin A (enterotoxin) and toxin B (cytotoxin)) (10), which are thought to be the primary causes of inflammation and colonic mucosal injury and it is remarkable that only pathogenic strains of *C. difficile* produce these toxins and cause clinical symptoms (11). Multiple methods for culturing *C. difficile* have been described in the literature (10-12). These studies have often been limited to two or three different variations in specimen processing or culture techniques (1). There are many different

Implication for health policy/practice/research/medical education:
C. difficileis photogenic bacteria usually present no symptoms but when it does present symptoms, they are sever ones like colitis. Antibiotic therapy is the most important risk factor for *C. difficile* colonization. About 1 in 100 hospitalized patients is diagnosed with the infection, and patients with *C. difficile* have a threefold increased risk of death during hospitalization. Therefore, early detection and prevention of this infection is essential.

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approaches that can be used for the laboratory diagnosis of *C. difficile*-associated diarrhoea (CDAD). The gold standard test for toxin B recognition with high sensitivity of 94-100% and specificity of 99%, is a tissue culturing assay for their cytotoxicity and utilization of pre incubation with neutralizing antibodies against this toxin which can detect as little as 10 picograms of toxin in stool, yet it is expensive and time consuming (takes 1-3 days). Another test, which has been developed to detect A and B toxins in stool samples is the enzyme linked immunosorbent assay (ELISA) with sensitivity of 66-94% and specificity of 92-98% (13-15). Furthermore, another method is glutamate dehydrogenase (GDH), which is characterized with a high level of sensitivity and a low level of specificity (16). Enzyme immunoassays rapidly detect toxins A and B, but their sensitivity varies greatly among various products (6). Toxigenic cultures and cytotoxin assays are considered as gold standard methods for the detection of toxigenic *C. difficile*, yet toxigenic cultures that combine anaerobic cultures and detect toxin A and B productions take at least 48 hours. Recently, new rapid molecular assays have been developed for the detection of genes encoding *C. difficile* toxin A (tcdA) and *C. difficile* toxin B (tcdB) directly in stool samples (17). For the PCR method, compared to the toxigenic culture, the sensitivity, specificity and positive and negative predictive values were 100%, 94.6%, 83.1%, and 100% respectively. The most significant advantage of the PCR assay is its rapidity and simplicity. In conclusion, the PCR assay is a reliable method for detecting toxigenic *C. difficile* from stool specimens and provides greater sensitivity than an enzyme immunoassay (18). The most important advantage of PCR in the clinical microbiology field is the rapidity that it offers for pathogen diagnosis.

2. Objectives

The purpose of this study was the isolation of *C. difficile* from stool samples and detection of A and B toxins encoding genes in order to serve as a routine method for clinical diagnosis.

3. Materials and Methods

3.1. Reference Strains

The Gram positive strain, *C. difficle* ATCC 10898 (19), was kindly provided by Dr. Aslani from the Research Center for Gastroenterology and Liver Disease, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

3.2. Sample Collection and Culture

A total 136 stool samples were collected from the teaching hospitals in the north of Tehran from January 2011 to September 2012.

3.3. Treatment Procedure

3.3.1. Methanol Shock Procedure

One lope of each stool sample was added to 2 mL of methanol (Merck, Germany). The mixture was vortexed, and incubated at room temperature for 2-3 minutes. After the methanol pre-treatment, a large drop was cultured in CCFA solid media and incubated at 37°C for 48-72 hours in an anaerobic atmosphere (Jar-GasPak system).

3.3.2. Yeast Shock Procedure

One lope of each stool sample was mixed with 2 mL of yeast extract broth. The mixture was vortexed and a large drop from it was cultured in CCFA solid media without centrifugation and incubated at 37°C for 48-72 hours in an anaerobic atmosphere (Ja-GasPak system).

3.3.3. Direct Plating Procedure

Using a Pasteur pipette (Fisher Scientific, Pittsburgh, PA, USA), a large drop of the stool that solved in PBS, was plated on solid media. The plates were incubated at 37°C for 48-72 hours in an anaerobic atmosphere (Jar-GasPak system).

3.4. Identification of C. difficile

Morphologic identification was detect by two method: 1) color created by UV light and 2) odor of colony. Colonies with typical morphology, under 365 nm UV fluorescence illumination and odor were identified as *C. difficil*e. The colonies were then identified with uniplex and multiplex PCR for A and B toxin encoding gene and cdd3 Spigagalia and Mastrantonio (20), Cohen et al. (21) as a species specific gene.

3.5. DNA Extraction

In order to extract DNA we used boiling methods. Briefly, One loop of bacterial colony was collected and added into 500 µL of DDW and centrifuged for 10 minutes at 13000 g, then the supernatant was discarded and 100 µL DDW was added to a plate, well mixed and boiled at 100°C for 20 minutes and centrifuged at 13000 g for 10 minutes to remove cell debris. The DNA containing supernatant t was used for amplification reactions. The quantity and quality of the extracted DNA was analyzed using NanoDrop 1000 (Thermo scientific, Japan) and gel electrophoresis, respectively.

3.6. Uniplex and Multiplex PCR

The sequences of the tcdA and tcdB genes of *C. difficile* were selected according to a study described by Spigagalia & Mastrantonio (20) and for the ccd3 gene instructions from the Cohen et al. (21) study were used. The specificity of each primer was investigated by performing BLAST in

the NCBI site. All the primers were constructed by CinnaClon Co, Iran, as shown in Table 1. The amplification products from the components of the uniplex and multiplex PCR, with a positive and negative control, were sequenced and thus determined to be correct. Separate PCR reactions were done for each primer in a final volume of 25 μL. The reaction contained 2 pmol of each primer (tcdA, tcdB, cdd3), 0.3 mM dNTPs mix (10 mM CinnaClon), and 0.3 U of Taq DNA polymerase (CinnaClon) in a PCR buffer (10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 2.5 mM $MgCl_2$). We also used positive (DNA of RIGLD strain 023) and negative controls (containing all PCR reagents without DNA) in our assay. PCR was performed in a BIORAD C1000TM thermal cycler with an initial denaturation step of 5 minutes at 95°C, then a touchdown procedure was implemented, consisting of 1 minute at 95°C, annealing for 1 minute at 52°C (tcdA, tcdB), 55°C (cdd3) and a final extension step at 72°C for 1 minute. A total of 30 cycles were performed. Afterwards, PCR products were electrophoresed and photographed under UV light with a Land camera (BIORAD, Universal hood II, USA). All PCR reactions were performed in triplicates.

3.7. Sensitivity of the Multiplex PCR Technique for tcdA, tcdB and cdd3 Genes

Performing a sensitivity test and determining the detection limits of *C. difficile* can define the strength of this test. The sensitivity of multiplex PCR assay was tested with a standard strain. After culturing the standard strain, genomic DNA was extracted by the method described above and tenfold serial dilutions were prepared from 100 ng to 10 pg, and multiplex-PCR was performed at each concentration.

4. Results

4.1. Comparison Between Methanol Shock and Yeast-Extract Enrichment

Our findings showed that, performing the initial shock by methanol and yeast-extract enrichment is better than direct plating methods. Additionally, it was expected that after methanol shock, bacterial growth gets faster than yeast extract enrichment. The optimal time for methanol shock was 48 hours but for the yeast extraction, enrichment requirement was a 72 hour incubation. The stool treatment method (none, methanol, yeast) had different effects on the *C. difficile* growth rate. According to findings of the present study, the most sensitive and effective recovery method for stool samples was the methanol shock treatment.

4.2. Clinical Findings

Among the 136 (100%) samples collected from an educational hospital located at north of Tehran, 75 (55.14%) were male and 61 (44.85%) were female. The samples' sex and age range are shown in Figure 1. Among the 136 (100%) samples, three (2.2%) were positive for toxigenic *C. difficile*. One (1.33%) out of the three (2.2%) positive toxigenic *C. difficile* samples was collected from a male candidate and two (3.27%) from females (Figure 2). Interestingly, among the three (2.2%) positive toxigenic *C. difficile* samples, two belonged to patients staying at the ICU and one was from a patient staying at the Children Clinical Center (Table 2). This corresponds to the high incidence of ICU-acquired diarrheas. During 2012, Cohen et al. (21) showed that median frequency of *C. difficile* is high in ICU patients. Clinical characterization of patients with *Clostridium difficile* is shown in Table 3.

4.3. Uniplex and Multiplex PCR

The presence of the tcdA, tcdB and cdd3 insertion sequences was assessed separately by the specific primer for each gene. The sizes of the tcdA, tcdB and cdd3 gene products were 624 bp, 412 bp and 622 bp, respectively (Figure 3). Between the 136 stool samples, three (2.2%) strains were isolated from specific cultures. Interestingly, all isolates contained tcdA, tcdB and cdd3 genes. tcdA and tcdB genes were detected with multiplex PCR (Figure 3).

Table 1. PCR Primers Sequences

Genes Detected	Amplicon Size, bp	Concentration, μM	References
TcdA			Spigagalia & Mastrantonio (20)
5'atgataaggcaacttcagtgg3'	624	0.1	
5'taagttcctcctgctccatcaa3'		0.1	
TcdB			Spigagalia & Mastrantonio (20)
5'gagctgcttcaattggagaga3'	412	0.1	
5'gtaacctactttcataacaccag3'		0.1	
Cdd3			Cohen et al. (21)
5'tccaatataataaattagcattcca3'	622	0.1	
5'ggctattacacgtaatccagata3'		0.1	

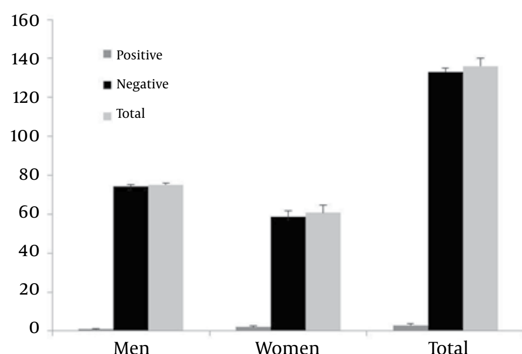


Figure 1. Frequency of Isolated Toxigenic *C. difficile*

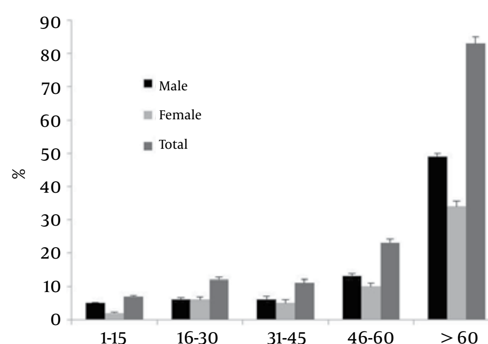


Figure 2. Frequency of Investigated Stool Samples for Toxigenic *C. difficile* on the Basis of Sex and Age

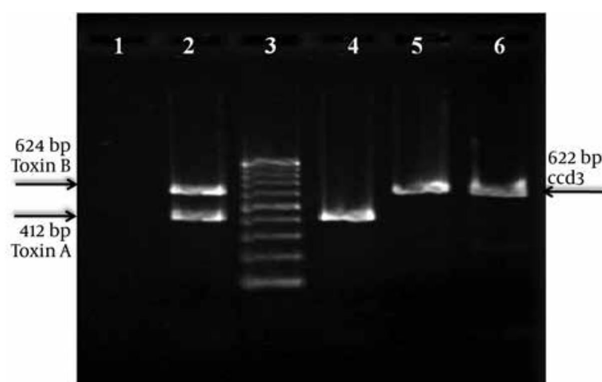
Table 2. Rate of Isolated toxigenic *C. difficile* in 136 Stool Samples from Baqiyatallah Hospital Wards

	Wards		
Toxigenic *C. difficile*	**ICU**	**Children Clinical Center**	**Outpatients**
Positive	2 (1.47%)	1 (0.735%)	0
Negative	5 (3.67%)	3 (2.205%)	125 (91.91%)
Total	7 (5.14%)	4 (2.94%)	125 (91.91%)

Table 3. Clinical Characterization of Patients with *Clostridium difficile*

	Age, y	Genus	Ward	Date of Hospitalization	Background Disease	Antibiotic Usage
1	10	M	children clinical center	26 day	chronic constipation, intestine surgery	Imipenem, Meropenem, Amikasin, Vancomycin, Clindamycin, Erythromycin
2	75	F	ICU	31day	cancer	Meropenem, Vancomycin, Tazocin
3	83	F	ICU	31day	DVT, UTI	Amikacin, Tazocin, Mmeropenem

Figure 3. Uniplex and Multiplex PCR Patterns of the Positive Strain



Lane 1, no template, control; Lane 2, multiplex PCR for A and B toxin encoding gene; lane 3, molecular weight standard (DNA molecular weight standard); lane 4, *C. difficile* toxin, B positive strain; lane 5, *C. difficile* toxin A, positive strain; lane 6, *C. difficile* ecdd3 insertion sequences, positive strain.

5. Discussion

People who carry bacterial pathogen the *C. difficile* usually have with no symptoms or on the other hand present severe symptoms such as colitis. Antibiotic therapy is the most important risk factor for *C. difficile* colonization (22). In 2010, Wachter showed that every one in 100 hospitalized patients is diagnosed with this infection, and patients with *C. difficile* had a threefold increased risk of death during their hospitalization (23). Over the past two decades, the prevalence *of C. difficile* has raised dramatically in medical centers as the leading cause of nosocomial infections and diarrhea following antibiotic therapy. (24). The incidence rate of *C. difficile* may be influenced by the presence of predisposing factors, such as type and duration of antimicrobial therapy, increased patient age, severity of underlying illnesses and length of hospital stay. Morbidity and mortality increases with the increased prevalence of *C. difficile* among hospitalized patients and places a significant economic burden on health services (25-27). *C. difficile* toxin B was isolated from 15 to 25% of antibiotic

associated diarrheas and more than 90% of patients pseudomembrane colitis (3-5). Since *C. difficile* has been known as the most common cause of nosocomial infections of the gastrointestinal tract, a variety of methods such as enzyme immunoassays (EIAs), or toxigenic cultures have been used as laboratory methods (28). On the other hand, between mediums, cycloserine - cefoxitin-fructose-agar (CCFA) has been recommended for the isolation of *C. difficile* (29). *C. difficile* in stool culture can be easily identified based on phenotypic characteristics such as colony morphology and under UV fluorescence light. Although, for precise identification additional tests, such as gas-liquid chromatographic analysis or even biochemical test panels (30) are required. In comparison to the Hink et al. study that used 1 hour for methanol treatment (31), we reduced this time to as few as 4 minutes, resulting in a more time saving method. Also, after methanol shock treatment, growth in the solid medium was faster, while treatment with yeast extract broth resulted in growth cessation on the solid medium. It was shown that methanol shock is more effective than yeast extract broth treatment. In the present work, we proposed to identify *C. difficile* by PCR amplification of tcdA (624 bp), tcdB (412 bp) (20) and uniplex PCR for cdd3 (622 bp) insertion sequences (21). The detection of non-toxigenic isolates by cdd3 amplification could also contribute to a better knowledge of the global epidemiology of this species. According to a previous study in Iran, *C. difficile* was isolated from 5.3% of patients with gastrointestinal complaints (32). In our study *C. difficile* infection was detected in 2.2% of stool samples, from which one strain (0.73%) was isolated from a patient at the Children Clinical Center and 2 strains (1.47%) were isolated from patients in the ICU. In other words, we set up a multiplex PCR for tcdA and tcdB detection. Findings of the present study are as follows:

1- Due to the constant extension, cdd3 insertion sequences among all strains of *C. difficile*, detection of this sequence can be applied for distinguishing between toxigenic and non-toxigenic *C. difficile* (21).

2- Ethanolic shock for separation of the *C. difficile* from fecal samples requires a shorter time than yeast extract enrichment method. It may be useful to rapidly screen for epidemic strains of *C. difficile*, in diagnostic microbiology laboratories. Further investigation and experimentation for the detection of *C. difficile* and toxins encoding genes by PCR, directly from stool samples is strongly recommended in order to save time and money. This study purpose was to 1- design an efficient method for isolation of *Clostridium difficile* from stool samples, 2- differentiate toxigenic and non toxigenic *C. difficile* from each other.

3- Detect A and B toxins encoding genes by PCR. One of our findings from this study was that ethanolic shock requires shorter time for separating *C. difficile* from fecal samples than yeast extract enrichment.

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Authors' Contribution

Sadegh Rahmati and Mohsen Rahimi performed the tests and protocols. Abbas Ali Imani Fooladi developed the original idea and supervised the Study. Jalil Fallah Mehrabadi and Mohammad Javad Soltanpour abstracted and analyzed the data. Raheleh Halabian and Hamid Sedighan wrote the manuscript and abstracted the data.

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The authors declare no financial disclosure.

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