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Molecular Characterization and Diagnosis of Nosocomial *Clostridium difficile* Infection in Hospitalized Patients

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Abstract

Background: Toxigenic *Clostridium difficile* is one of the prevalent diarrheagenic pathogens in hospitalized patients.
Objectives: The study assessed the ability of three diagnostic methods in identifying *C. difficile* strains. The genotyping of the isolates was done, as well.
Methods: Stool samples were subjected to three different diagnostic methods including direct stool culture, glutamate dehydrogenase enzyme immunoassay (GDH-EIA), and direct stool PCR for the detection of the *tcd*A and *tcd*B genes. The sensitivity and specificity of the tests were evaluated. The genotyping was done by the PFGE method.
Results: Of 120 samples, 20 (16%) were positive for *C. difficile* based on PCR, while 15 (12.5%) and 12 (10%) were positive according to GDH-EIA and direct stool culture. Among patients with *C. difficile*-associated diseases (CDAD), 11 (61%) were more than 65-years-old. The specificity of PCR, GDH-EIA, and direct culture was almost similar and equal to 100%, but their sensitivity was 90%, 70%, and 60%, respectively. The positive predictive value (PPV) was lower for GDH-EIA than for the other two methods, and the highest negative predictive value (NPV) was related to the PCR method. The results showed a high similarity between the isolates, and only were three pulsotypes differentiated among the isolates.
Conclusions: The specificity and sensitivity of the direct stool PCR method were higher than those of the other two methods. Although PCR inhibitors may reduce its ability for the correct diagnosis of negative samples, it seems to be a reliable method for the detection of *C. difficile* infection. The weakness of the GDH-EIA method was its lower PPV, which can cause false-positive results. Toxin patterns and pulsotypes of *C. difficile* isolates revealed a high similarity between the strains isolated from the same units.

Keywords: *Clostridium difficile*, GDH-EIA, CDI, Nosocomial Infection, PFGE

1. Background

Toxigenic *Clostridium difficile* is one of the primary diarrheagenic pathogens in hospitalized patients. Although *C. difficile* can cause pseudomembranous colitis (PMC), it is found in the normal fecal flora of 5% of healthy adults (1). Other infections like antibiotic-associated colitis, antibiotic-associated diarrhea, and non-antibiotic-associated diarrhea (2, 3) showed to be associated with *C. difficile*. This bacterium is on the top list of agents causing healthcare-associated infections (HIAs). The intensity of *C. difficile* infection (CDI) ranges from mild diarrhea to PMC, which is caused by toxigenic strains and can lead to death (4). The CDI has different risk factors, including hospitalization, old age, and exposure to antibiotics such as β-lactams, clindamycin, and cephalosporins (5).

Healthcare-associated infections are considered a noticeable threat to public health not only in developing countries but also in developed ones such as the United States and Europe (6). The pulsotype (NAP1)/ribotype 027 of *C. difficile* emerged in North America in 2003, and it has been detected as a cause of CDI prevalence in the United States and Canada (7-9). Since then, CDI has shown an upward trend among hospitalized patients in a way that roughly half a million cases and 29,000 deaths were reported in 2012. Two-thirds of these cases were associated with hospitalized patients, and the healthcare incidence was 92.8 per 100,000 persons (10). In addition to the North

American countries facing this public health threat, the European countries are involved as well, as ribotype 078 was responsible for CDI prevalence in Europe in 2005 (11). The incidence rate of CDI in Europe has been reported with high variations. In a survey conducted in all European countries in 2008, the average incidence rate of *C. difficile* was reported as 4.1 per 100,000 patient-days per hospital, in the range of 0.0 to 36.3 (12). Although the threat of CDI among hospitalized patients is rapidly increasing worldwide, the accurate estimation of this lethal infection in developing countries is still vague due to the lack of precise and exact diagnostic and surveillance protocols.

The differential diagnosis of diarrhea in hospitalized patients is important for choosing the treatment; for instance, *C. difficile* is more likely to be the cause of infection than other enteric pathogens in patients with diarrhea arising after 72 h of hospitalization (13). In this organism, there are different virulence factors responsible for *C. difficile*-associated diarrhea (CDAD) such as enterotoxins, ADP-ribosylating toxin, and spore formation ability (14). Recent research determined that two exotoxins of toxin A and toxin B (cytotoxin) are mainly associated with primary colonic mucosal injury and inflammation (15). Toxin A is an enterotoxin that can cause fluid secretion and consequently, diarrhea and also act as a chemotactic agent for neutrophils and cytokines secretion. Toxin B has a strong cytotoxicity ability that can be lethal for many cell lines (16). Many studies revealed that almost all clinical isolates produced one or both toxins. It is evidenced that rapid, accurate diagnosis of *C. difficile* is a critical key in the CDI control and prevention. In recent studies, different diagnostic methods have been developed for the detection of *C. difficile* isolates in clinical samples such as direct culture, immunoassay, molecular detection, and cell culture. The key to the diagnosis of these bacteria is the power of the selected method to differentiate toxigenic strains from non-toxigenic strains. Although the cell cytotoxicity assay (cell culture) is the "gold standard" method for the identification of toxigenic strains, it is not time- and cost-effective for the Healthcare System and Medical Laboratory Section (17, 18). Another approach to the detection of *C. difficile* strains in clinical samples is the use of immunoassay methods, including enzyme-linked immunosorbent assay (19), which is used to detect toxins and other enzymes produced in the strains. For source tracking of CDI, the clinical strains should be analyzed by molecular typing methods. The PFGE is the standard gold method for strain genotyping, which can determine the genetic relationship among strains with a high confidence level (20).

2. Objectives

In the present study, we determined the prevalence of *C. difficile* strains among hospitalized patients at a teaching hospital of Iran University of Medical Sciences, Tehran, Iran, by using the PCR method to specify the toxigenic pattern. Besides, three different methods were evaluated to select the most reliable one for the identification of *C. difficile* isolates. Finally, the genetic relationship among *C. difficile* strains isolated from stool samples of patients was determined by the PFGE technique.

3. Methods

3.1. Sample Collection

In this study, 120 stool samples were collected from hospitalized patients at a teaching hospital of Iran University of Medical Sciences, Tehran, Iran, from February 2016 to January 2017. The inclusion criteria were defined as having diarrhea symptoms, mid-term term or long-term hospitalization (three days or more), taking antibiotics during hospitalization, or having operations (21). The diarrhea was diagnosed by a watery, loose, bloody, or mucoid stool with at least three times a day frequency. The patients were divided into three groups, including children and adolescents (1-19-years-old), young and middle-aged individuals (20-64-years-old), and the elderly (more than 65-years-old).

All stool samples were transferred to the Antimicrobial Resistance Research Center and stored under the conditions of the cold chain (4°C) for 4 h after sampling. The samples were subjected to three different diagnostic methods, including (1) direct stool culture; (2) Glutamate dehydrogenase enzyme immunoassay (GDH-EIA) for *C. difficile* using *Clostridium K*-SeT commercial kit (Coris BioConcept, Belgium); and (3) direct stool PCR for the detection of *tcd*A and *tcd*B genes.

3.2. Stool Culture and Bacterial Isolation

The isolation of *C. difficile* strains was conducted following the alcohol shock protocol (22). According to previous studies, 1 mL (or 1 g) of stool specimen was treated with 1 mL absolute ethanol and incubated at room temperature for 2 min, followed by culturing on cycloserine cefoxitin fructose agar (CCFA) and pre-poured chromID agar (Biomerieux SA, France) (23). The CCFA culture media were supplemented with 7% defibrinated horse blood, 0.1% sodium taurocholate, 250 $\mu g.mL^{-1}$ cycloserine, 10 $\mu g.mL^{-1}$ cefoxitin, and 250 $\mu g.mL^{-1}$ amphotericin B to enhance the germination of *C. difficile* spores and prevent the growth of

other bacteria and fungi. Another 1 mL (or 1 g) of stool specimen was mixed with 1 mL of 5% yeast extract and then immediately cultured on the CCFA media to prevent the missing of *C. difficile* strains. All cultured media were incubated in anaerobic conditions using a jar with a gas pack (Anaerocult A, Merck, Germany) at 37°C. Plates were monitored for five days and the incubation of negative cultures continued for seven days. Then, *C. difficile* colonies having an irregular edge and the odor of horse manure with Gram-positive reactions were identified as large colonies. The gray to black colonies after 24 h on Chrom ID were identified as positive colonies. Conventional biochemical tests were performed to confirm the isolated strains from the specific media. To finalize the diagnosis, motile strains that were positive for producing gelatinase and H_2S and negative for catalase, oxidase, and indole were confirmed as *Clostridium difficile*.

3.3. Glutamate Dehydrogenase Enzyme Immunoassay for C. difficile

Glutamate dehydrogenase is a metabolic enzyme that has recently shown to play a critical role in the rapid diagnosis of *C. difficile*, as all strains produce a high amount of this enzyme. *Clostridium K*-SeT (Coris BioConcept, Belgium) was used to direct detection of *C. difficile* strains from stool samples. The cassettes were made of a nitrocellulose membrane coated with an antibody (GDH) directed against *C. difficile* antigen. The dilution buffer containing Tris, EDTA, and NaN_3 (< 0.1%) worked as a detergent and protein blocker. All stool samples were tested with *Clostridium K*-SeT according to the manufacturer's instruction. The samples were recorded as positive when a reddish-purple line appeared across the control (C) and test (T) lines on the cassettes.

3.4. Direct PCR Amplification of Toxin Genes Sequences

Total DNA was extracted from fecal samples using QIAamp DNA Stool Minikit (Qiagen, Germany) and used as the DNA template in PCR. Two separate PCRs were carried out using specific primers for the *tcd*A and *tcd*B genes (Table 1). The PCR mixture consisted of a final volume of 25 μL containing 12.5 μL ready-to-use master mix (including $MgCl_2$, dNTP, and Taq enzyme) (SinaClon BioScience Co. Iran), 0.25 μL of 10 nmol.μL^{-1} of each primer, 5 μL of extracted DNA, and sterile nuclease-free water. The PCR program started by an initial denaturation at 93°C for 5 min, followed by 30 cycles including denaturation at 95°C for one minute, annealing at 52°C for one minute, extension at 72°C for one minute, and a final extension at 72°C for 5 min; finally, the reaction was held at 4°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with commercial DNA safe stain (SinaClon BioScience Co. Iran). The images were captured using the UVItec gel documentation system (Cleaver Scientific Ltd., United Kingdom)

Table 1. Primers Used in This Study

Gene	Primer sequence (5' - 3')	Amplicon Size	Reference
tcdA	Forward: GGAAGAAAAGAACTTCTG-GCTCACTCAGGT	251	(24)
	Reverse: CCCAATAGAAGATTCAATATTAAGCTT		
tcdB	Forward: GGTGGAGCTGCTTCATTGGAGAG	418	(25)
	Reverse: GTGTAACCTACTTTCATAACACCA		

3.5. Evaluation of Diagnostic Methods Efficiency

The efficiency of diagnostic methods was evaluated by interpreting the test results. In this survey, if the results of two out of three different methods were positive, the sample would be considered a true positive. Also, four different indices, including specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for PCR, direct culture (DC), and GDH-EIA assay. The PPV is the probability that a positive test result is truly positive and the NPV is the probability that a negative test result is truly negative. They were calculated as follows: PPV = [true positive/true positive + false positive] × 100, NPV = [true negative/true negative + false negative] × 100, sensitivity = [true positive/true positive + false negative] × 100, and specificity = [true negative/true negative + false negative] × 100 (26).

3.6. PFGE Analysis of the Isolates

The genotyping of isolates was done according to the PulseNetprotocol for *C. botulinum* with some modification specified for *C. difficile* strains. Briefly, all isolates were cultured on CCFA supplemented with blood, antibiotics, and sodium taurocholate and incubated anaerobically for 48 h. Bacterial suspensions were made with 1 McFarland turbidity. Cells were washed twice with 1,000 μL cell suspension buffer. The washed cells were inoculated on Egg Yolk agar under anaerobic conditions. The grown colonies were suspended in 1.5 mL of lysis buffer (12 mM Tris, 2 M NaCl, 200 mM EDTA, 1% Brij 58, 0.4% deoxycholate, and 1% Sarcosyl) until OD_{600} reached 0.8 - 1. The bacterial suspension was centrifuged, the supernatant was removed, and the pellet was re-suspended in 400 μL of lysis buffer, proteinase K (0.665 mg.mL^{-1}), lysozyme (4 mg.mL^{-1}), and 20U mutanolysin and incubated in 55°C water bath for 20 - 30

min. Melted 1.2% SeaKem Gold agarose (400 μL) was added to cell suspensions and mixed gently. The mixture was immediately poured into the PFGE mold. Plugs were washed using sterile ES buffer (10 mM Tris-HCl [pH = 7.5], 10 mM Na_2EDTA, plus proteinase K [0.14 $mg.mL^{-1}$]), incubated in 55°C shaker water bath for at least 2 h, and preheated in 55°C ultrapure sterile water two times; each time of shaking was in the water bath at 55°C for 15 min. In the next step, the plugs were washed six times with preheated (55°C) sterile TE buffer. Each plug was cut into four pieces, each of which was digested in one PFGE run. The sample plugs were digested with 30 U *Sma*I restriction enzyme and 1 μg of RNaseA and incubated at 25°C for at least one hour. Then, the standard plugs (*Salmonella* ser. *Braenderup* H9812) were digested with *Xba*I and incubated at 37°C to use as a DNA size marker. The digested DNA fragments were separated in 1.5% agarose in 0.5X TBE buffer with 200 μM thiourea and electrophoresed for 22 h at 14°C with an initial switch time of one second, final switch time of 35 s, and the gradient of 6 $V.cm^{-1}$. The isolates were classified in the same pulsotype if they showed more than 80% similarity in their patterns (27).

3.7. Statistical Analysis

The statistical analysis was done with SPSS version 21 software. The BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the PFGE patterns. The patterns were compared using the Dice coefficient and unweighted pair group method with arithmetic averages (UPGMA) clustering. A dendrogram was constructed using an optimization value of 0.5% and a position tolerance of 1.0%.

4. Results

During 12 months, 120 stool samples were collected from patients in this study. All patients had a history of surgical operation, infectious diseases, or antibiotic therapy. A total of 18 (15%) out of 120 samples were positive for the presence of *C. difficile* strains according to the molecular diagnosis results, while 15 (12.5%) and 12 (10%) cases were positive according to the GDH-EIA and direct stool culture, respectively. Eight (16.3%) out of 49 women and 10 (14.1%) out of 71 men were identified as CDAD-positive. Among patients with CDAD infection, 11 (61%) cases had more than 65-years-old, which was significantly different from the other two age groups ($P < 0.05$) (Table 2). All positive patients (except for one case) had been treated with at least two or more types of antibiotics, especially fluoroquinolones and β-lactams. The exceptional case was hospitalized in the Medical Intensive Care Unit (MICU) with the diagnosis of chronic obstructive pulmonary disease (COPD) that had taken only ceftriaxone before sampling. Also, 12 (60%) out of 20 cases had received ciprofloxacin, five (25%) cases metronidazole, six (30%) cases vancomycin, and eight (40%) cases β-lactams including cephalosporins and carbapenems.

Table 2. Frequency of Positive Samples for *Clostridium difficile* in Different Age Groups and Genders[a]

Gender	1 - 19 Years	20 - 64 Years	> 65 Years
Female	2 (11.1)	1 (5.5)	5 (27.7)
Male	3 (16.6)	1 (5.5)	6 (33.4)
Total	5 (27.7)	2 (11.1)	11 (61.2)

[a]Values are expressed as No. (%).

4.1. Diagnosis of CDAD with PCR

The whole genome was analyzed in stool samples for toxin detection using *tcd*A and *tcd*B specific primers. According to the results of PCR amplification, 251 and 418 bp DNA bands (Figure 1) related to the *tcd*A and *tcd*B genes were present in 17 (94%) and 16 (89%) isolates, respectively (Table 3).

Table 3. Toxin Patterns of *Clostridium difficile* Isolated From Diarrheal Patients[a]

Toxin Gene Pattern	Values
***tcdA*$^+$, *tcdB*$^+$**	15 (83.5)
***tcdA*$^+$, *tcdB*$^-$**	2 (11)
***tcdA*$^-$, *tcdB*$^+$**	1 (5.5)
Total	18 (100)

[a]Values are expressed as No. (%).

4.2. Efficiency of Diagnostic Methods

The sensitivity (the ability of a test to classify correctly a sample as positive), specificity (the ability of a test to classify a sample correctly as negative), NPV, and PPV indices of direct culture, GDH-EIA, and PCR assay are shown in Table 4. The PCR and direct culture methods had the highest accuracy to detect the negative samples. The main problem was the detection of true positive samples, as the sensitivity of direct culture and EIA-DGH assay was 60% and 70%, respectively (Table 4).

4.3. Genotyping of the Isolates

Among 18 positive samples, only 12 *C. difficile* strains were isolated via direct culture, and they all were subjected to the PFGE assay. The fingerprints with 8 to 11 bands were

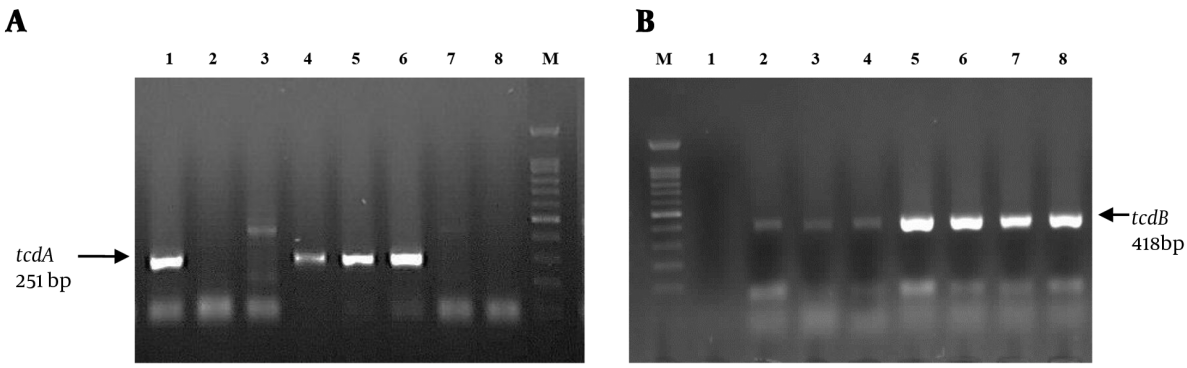


Figure 1. PCR amplification of *tcd*A and *tcd*B genes among patients with CDAD. A, Amplification of *tcdA*; M: 1 kb DNA size marker; lane 1, confirmed *C. difficile* clinical isolate used as a positive control; lane 2, *E. coli* ATCC 2599 as a negative control; lanes 3, 7, and 8, negative samples; lanes 4 - 6, positive samples for *tcd*A; B, amplification of tcdB; M: 1 kb DNA size marker; lane 1, *E. coli* ATCC 2599 as a negative control; lane 2, confirmed *C. difficile* clinical isolate used as a positive control; lanes 3 - 8, positive samples.

Table 4. The Efficiency of Direct Culture and GDH-EIA Method in the Diagnosis of *Clostridium difficile*

Assay	Number of Positive Samples	Number of Negative Samples	Specificity, %	Sensitivity, %	PPV	NPV
PCR	18	102	100	90	100	98
Direct culture	12	108	100	60	100	92
GDH-EIA	15	105	99	70	93	94

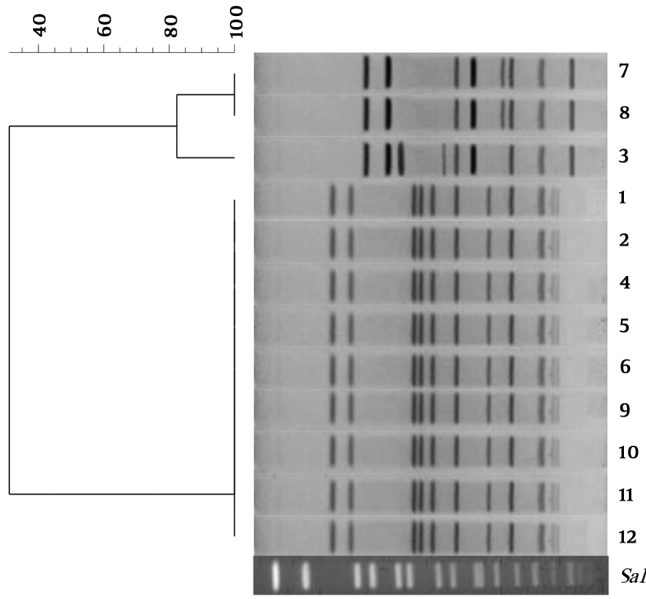
detected in each isolate. The results showed a high similarity between the isolates and only were three pulsotypes (named as PF-A, PF-B, and PF-C) differentiated from the isolates (Figure 2). The PF-A pulsotype was the most common pulsotype, determined in nine (75%) isolates, all of which were isolated from hospitalized patients in the internal ward. The PF-B pulsotype was related to two patients who were admitted to the neurological ward. The PF-Cpulsotype was determined in only one patient hospitalized in the MICU. As shown in Figure 2, a correlation was detected between the pulsotypes and toxinotypes.

5. Discussion

As known, *C. difficile* is part of the normal intestinal flora. It is considered an opportunistic pathogen during the usage of antibiotics or surgical operations (28). Toxigenic strains of *C. difficile* can cause fatal infection and nowadays, are the main nosocomial pathogens. According to the studies, CDI is the cause of 10% - 20% of all antibiotic-associated diarrhea cases and all colitis cases, occurring as the consequences of antibiotic therapy worldwide (29). Determining the accurate prevalence rate and genetic profiles and using a quick, reliable method to identify *C. difficile* strains play a critical role in controlling and preventing CDI in hospitals and healthcare settings.

According to previous studies conducted in Europe, America, and some Asian countries, the prevalence and epidemiological profile of *C. difficile* are changing, and they are completely different based on geographical disparities, the type of therapies used by clinicians, and the different genetic properties of *C. difficile* isolates (1, 10, 30). This has been reported as a major risk for nosocomial infection control in a few studies conducted to determine the regional prevalence and genotype patterns of *C. difficile* strains in Iranian hospitals as hospital acquired *Clostridium difficile* infection (HA-CDI) (31, 32). In the present study, the prevalence rate of HA-CDI was 15% among hospitalized patients, which is almost consistent with the findings of other studies by Goudarzi et al. (31) and Jalali et al. (33), in which the prevalence rate was reported as 21% and 20%, respectively. In the current study, an upward trend was observed in the prevalence of *C. difficile* among hospitalized patients in Iran, as the prevalence of *C. difficile* reported by Sadeghifard et al. (17) in 2005 was about 10.3%, only 6.1% of which were toxigenic strains. This upward trend has been reported in other studies conducted in Europe and the United States (30, 34), in which not only the incidence of CDI among hospitalized patients but also the community-acquired CDI (CA-CDI) has been reported to be raising (35).

Based on other reports, CDI disproportionately affects elderly patients (36). The results of this study asserted the hypothesis of old age being as a risk factor for CDI, as 61%



Pulsotype	Toxinotype	Hospital ward	N of isolates
PF-B	*tcd*A^{+}, *tcd*B^{-}	Neurological ward	2
PF-C	*tcd*A^{-}, *tcd*B^{+}	MICU	1
PF-A	*tcd*A^{+}, *tcd*B^{+}	Internal ward	9

Figure 2. Dendrogram of genetic relationships among *Clostridium difficile* isolates and associated toxin patterns

of patients with positive PCR tests for *C. difficile* were more than 65-years-old. However, contrary results were observed in some studies. In a study conducted by Jalali et al. (33), less than 30% of CDI patients were over 65-years-old, and most of the positive patients were younger than 43 years. Different diagnostic methods have been modified to the approach of rapid and accurate diagnosis of CDI (16, 37, 38). According to the present study results, the direct stool PCR toxin assay showed the highest sensitivity and specificity among other methods. The commercial *Clostridium K*-SeT (Coris BioConcept, Belgium) produced based on the EIA-GDH assay showed the potential of this method to use as a reliable and rapid alternative for direct culture. The sensitivity of this method was higher than that of direct stool culture, indicating that this method can detect positive samples more accurately than direct culture does. The specificity of both methods was almost 100%, indicating that both have a great ability to recognize negative samples, but direct culture wrongly detects positive samples as negative ones. This disparity occurs because of the external errors during direct culture, including technician, material, or instrument errors. *Clostridium K*-SeT can be used for the rapid detection of *C. difficile* strains from stool samples but the PCR toxin assay should be done for the final confirmation of toxigenic strains. The sensitivity of the PCR technique in detecting low amounts of bacterial DNA in samples and reducing external errors relative to other methods introduces PCR as a reliable method for the diagnosis of bacterial strains, especially in the case of toxigenic *C. difficile* strains.

The PFGE method was performed for *C. difficile* isolates genotyping. Different typing methods have been used for *C. difficile* genotyping (39, 40) but PFGE has been introduced as a Gold standard (41). Three different pulsotypes (PF-A, PF-B, and PF-C) were recognized among the isolates, each of which was related to a different toxin pattern, indicating the effectiveness of PFGE in recognizing genetics contents (Figure 2). The PF-A pulsotype associated with strains isolated from internal ward patients had the *tcd*A^{+}/*tcd*B^{+} toxin pattern and it was the most common pulsotype (75%). The PF-B pulsotype associated with strains isolated from neurological ward patients had the *tcd*A+/*tcd*B- toxin profile. The PF-C pulsotype associated with one strain isolated from a patient with COPD hospitalized in the MICU showed the *tcd*A^{-}/*tcd*B^{+} toxin pattern. Some studies showed that COPD could encounter CDI, but some others claimed that CDI could increase in COPD patients because of early antibiotic administration. The internal and neurological wards were located in different parts of the hospital and it could be the reason for patients to be infected with different colons of *C. difficile*. It is worth noting that patients in the same ward had HA-CDI caused by the clonally related isolates. The transmission of CDI among patients is a critical threat in nosocomial infection control, which should be prevented by using high-level hygiene protocols.

5.1. Conclusions

The PCR toxin assay is a reliable method for the accurate diagnosis of CDI. Although the EIA-GDH assay has a lower sensitivity, it can be used for rapid screening and the results should be confirmed by molecular methods. In addition, the toxin patterns and genotypes of *C. difficile* isolates were compatible with each other and provided essential data for source tracking and controlling CDI distribution.

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Footnotes

Authors' Contribution: AM supervised the study. MB designed, supervised, and performed the laboratory steps and wrote the manuscript. SR collected the samples. BB analyzed the molecular data. MR was microbial consultant. PK analyzed the PFGE results. AD was language editor. MA performed microbial diagnostic tests.

Conflict of Interests: There is no conflict of interest to declare.

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References

1. Lessa FC, Gould CV, McDonald LC. Current status of Clostridium difficile infection epidemiology. *Clin Infect Dis*. 2012;**55 Suppl 2**:S65–70. doi: 10.1093/cid/cis319. [PubMed: 22752867]. [PubMed Central: PMC3388017].
2. Knoop FC, Owens M, Crocker IC. Clostridium difficile: clinical disease and diagnosis. *Clin Microbiol Rev*. 1993;**6**(3):251–65. doi: 10.1128/cmr.6.3.251. [PubMed: 8358706]. [PubMed Central: PMC358285].
3. Brettle RP, Poxton IR, Murdoch JM, Brown R, Byrne MD, Collee JG. Clostridium difficile in association with sporadic diarrhoea. *Br Med J (Clin Res Ed)*. 1982;**284**(6311):230–3. doi: 10.1136/bmj.284.6311.230. [PubMed: 6799113]. [PubMed Central: PMC1495803].
4. Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva JJ. Clostridium difficile-associated diarrhea and colitis. *Infect Control Hosp Epidemiol*. 1995;**16**(8):459–77. doi: 10.1086/648363. [PubMed: 7594392].
5. Bartlett JG. Clinical practice. Antibiotic-associated diarrhea. *N Engl J Med*. 2002;**346**(5):334–9. doi: 10.1056/NEJMcp011603. [PubMed: 11821511].
6. Balsells E, Shi T, Leese C, Lyell I, Burrows J, Wiuff C, et al. Global burden of Clostridium difficile infections: A systematic review and meta-analysis. *J Glob Health*. 2019;**9**(1):10407. doi: 10.7189/jogh.09.010407. [PubMed: 30603078]. [PubMed Central: PMC6304170].
7. MacCannell DR, Louie TJ, Gregson DB, Laverdiere M, Labbe AC, Laing F, et al. Molecular analysis of Clostridium difficile PCR ribotype 027 isolates from Eastern and Western Canada. *J Clin Microbiol*. 2006;**44**(6):2147–52. doi: 10.1128/JCM.02563-05. [PubMed: 16757612]. [PubMed Central: PMC1489423].
8. McDonald LC, Killgore GE, Thompson A, Owens RJ, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of Clostridium difficile. *N Engl J Med*. 2005;**353**(23):2433–41. doi: 10.1056/NEJMoa051590. [PubMed: 16322603].
9. Burke KE, Lamont JT. Clostridium difficile infection: A worldwide disease. *Gut Liver*. 2014;**8**(1):1–6. doi: 10.5009/gnl.2014.8.1.1. [PubMed: 24516694]. [PubMed Central: PMC3916678].
10. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, et al. Burden of Clostridium difficile infection in the United States. *N Engl J Med*. 2015;**372**(9):825–34. doi: 10.1056/NEJMoa1408913. [PubMed: 25714160].
11. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, et al. Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis*. 2008;**47**(9):1162–70. doi: 10.1086/592257. [PubMed: 18808358].
12. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. Clostridium difficile infection in Europe: A hospital-based survey. *Lancet*. 2011;**377**(9759):63–73. doi: 10.1016/S0140-6736(10)61266-4. [PubMed: 21084111].
13. Siegel DL, Edelstein PH, Nachamkin I. Inappropriate testing for diarrheal diseases in the hospital. *JAMA*. 1990;**263**(7):979–82. [PubMed: 2299766].
14. Bagdasarian N, Rao K, Malani PN. Diagnosis and treatment of Clostridium difficile in adults: A systematic review. *JAMA*. 2015;**313**(4):398–408. doi: 10.1001/jama.2014.17103. [PubMed: 25626036]. [PubMed Central: PMC6561347].
15. Gerding DN, Olson MM, Peterson LR, Teasley DG, Gebhard RL, Schwartz ML, et al. Clostridium difficile-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch Intern Med*. 1986;**146**(1):95–100. [PubMed: 3942469].
16. Yucesoy M, McCoubrey J, Brown R, Poxton IR. Detection of toxin production in Clostridium difficile strains by three different methods. *Clin Microbiol Infect*. 2002;**8**(7):413–8. doi: 10.1046/j.1469-0691.2002.00440.x. [PubMed: 12199851].
17. Sadeghifard N, Salari MH, Ghassemi MR, Shirazi MH, Feizabadi MM, Kazemi B, et al. Prevalence of Clostridium difficile-associated diarrhea in hospitalized patients with nosocomial diarrhea. *Iran J Public Health*. 2005:67–72.
18. Turgeon DK, Novicki TJ, Quick J, Carlson L, Miller P, Ulness B, et al. Six rapid tests for direct detection of Clostridium difficile and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J Clin Microbiol*. 2003;**41**(2):667–70. doi: 10.1128/jcm.41.2.667-670.2003. [PubMed: 12574264]. [PubMed Central: PMC149656].
19. Terhes G, Urban E, Soki J, Hamid KA, Nagy E. Community-acquired Clostridium difficile diarrhea caused by binary toxin, toxin A, and toxin B gene-positive isolates in Hungary. *J Clin Microbiol*. 2004;**42**(9):4316–8. doi: 10.1128/JCM.42.9.4316-4318.2004. [PubMed: 15365032]. [PubMed Central: PMC516352].
20. Samie A, Obi CL, Franasiak J, Archbald-Pannone L, Bessong PO, Alcantara-Warren C, et al. PCR detection of Clostridium difficile triose phosphate isomerase (tpi), toxin A (tcdA), toxin B (tcdB), binary toxin (cdtA, cdtB), and tcdC genes in Vhembe District, South Africa. *Am J Trop Med Hyg*. 2008;**78**(4):577–85. [PubMed: 18385352].
21. Bartlett JG, Gerding DN. Clinical recognition and diagnosis of Clostridium difficile infection. *Clin Infect Dis*. 2008;**46 Suppl 1**:S12–8. doi: 10.1086/521863. [PubMed: 18177217].
22. Riley TV, Brazier JS, Hassan H, Williams K, Phillips KD. Comparison of alcohol shock enrichment and selective enrichment for the isolation of Clostridium difficile. *Epidemiol Infect*. 1987;**99**(2):355–9. doi: 10.1017/s0950268800067832. [PubMed: 3315708]. [PubMed Central: PMC2249267].

23. Carson KC, Boseiwaqa LV, Thean SK, Foster NF, Riley TV. Isolation of Clostridium difficile from faecal specimens–a comparison of chromID C. difficile agar and cycloserine-cefoxitin-fructose agar. *J Med Microbiol*. 2013;**62**(Pt 9):1423–7. doi: 10.1099/jmm.0.056515-0. [PubMed: 23579394].
24. Kato N, Ou CY, Kato H, Bartley SL, Brown VK, Dowell VJ, et al. Identification of toxigenic Clostridium difficile by the polymerase chain reaction. *J Clin Microbiol*. 1991;**29**(1):33–7. [PubMed: 1993763]. [PubMed Central: PMC269697].
25. Cohen SH, Tang YJ, Silva JJ. Analysis of the pathogenicity locus in Clostridium difficile strains. *J Infect Dis*. 2000;**181**(2):659–63. doi: 10.1086/315248. [PubMed: 10669352].
26. Wong HB, Lim GH. Measures of diagnostic accuracy: Sensitivity, specificity, PPV and NPV. *Proceed Singapore Healthcare*. 2011;**20**(4):316–8. doi: 10.1177/201010581102000411.
27. Martin H, Willey B, Low DE, Staempfli HR, McGeer A, Boerlin P, et al. Characterization of Clostridium difficile strains isolated from patients in Ontario, Canada, from 2004 to 2006. *J Clin Microbiol*. 2008;**46**(9):2999–3004. doi: 10.1128/JCM.02437-07. [PubMed: 18650360]. [PubMed Central: PMC2546775].
28. Hsu M, Wang J, Huang W, Liu Y, Chang S. Prevalence and clinical features of Clostridium difficile-associated diarrhea in a tertiary hospital in Northern Taiwan. *J Microbiol Immunol Infect*. 2006;**39**(3):242–8.
29. Shaughnessy MK, Amundson WH, Kuskowski MA, DeCarolis DD, Johnson JR, Drekonja DM. Unnecessary antimicrobial use in patients with current or recent Clostridium difficile infection. *Infect Control Hosp Epidemiol*. 2013;**34**(2):109–16. doi: 10.1086/669089. [PubMed: 23295554].
30. Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, et al. The changing epidemiology of Clostridium difficile infections. *Clin Microbiol Rev*. 2010;**23**(3):529–49. doi: 10.1128/CMR.00082-09. [PubMed: 20610822]. [PubMed Central: PMC2901659].
31. Goudarzi M, Goudarzi H, Alebouyeh M, Azimi Rad M, Shayegan Mehr FS, Zali MR, et al. Antimicrobial susceptibility of clostridium difficile clinical isolates in iran. *Iran Red Crescent Med J*. 2013;**15**(8):704–11. doi: 10.5812/ircmj.5189. [PubMed: 24578839]. [PubMed Central: PMC3918196].
32. Khoshdel A, Habibian R, Parvin N, Doosti A, Famouri F, Eshraghi A, et al. Molecular characterization of nosocomial Clostridium difficile infection in pediatric ward in Iran. *Springerplus*. 2015;**4**:627. doi: 10.1186/s40064-015-1268-0. [PubMed: 26543762]. [PubMed Central: PMC4628048].
33. Jalali M, Khorvash F, Warriner K, Weese JS. Clostridium difficile infection in an Iranian hospital. *BMC Res Notes*. 2012;**5**:159. doi: 10.1186/1756-0500-5-159. [PubMed: 22436392]. [PubMed Central: PMC3317812].
34. McDonald LC, Owings M, Jernigan DB. Clostridium difficile infection in patients discharged from US short-stay hospitals, 1996-2003. *Emerg Infect Dis*. 2006;**12**(3):409–15. doi: 10.3201/eid1205.051064. [PubMed: 16704777]. [PubMed Central: PMC3291455].
35. Depestel DD, Aronoff DM. Epidemiology of Clostridium difficile infection. *J Pharm Pract*. 2013;**26**(5):464–75. doi: 10.1177/0897190013499521. [PubMed: 24064435]. [PubMed Central: PMC4128635].
36. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). *Infect Control Hosp Epidemiol*. 2010;**31**(5):431–55. doi: 10.1086/651706. [PubMed: 20307191].
37. Keessen EC, Hopman NE, van Leengoed LA, van Asten AJ, Hermanus C, Kuijper EJ, et al. Evaluation of four different diagnostic tests to detect Clostridium difficile in piglets. *J Clin Microbiol*. 2011;**49**(5):1816–21. doi: 10.1128/JCM.00242-11. [PubMed: 21411571]. [PubMed Central: PMC3122649].
38. Darkoh C, Dupont HL, Kaplan HB. Novel one-step method for detection and isolation of active-toxin-producing Clostridium difficile strains directly from stool samples. *J Clin Microbiol*. 2011;**49**(12):4219–24. doi: 10.1128/JCM.01033-11. [PubMed: 21976761]. [PubMed Central: PMC3232957].
39. Rupnik M, Brazier JS, Duerden BI, Grabnar M, Stubbs SLJ. Comparison of toxinotyping and PCR ribotyping of Clostridium difficile strains and description of novel toxinotypes. *Microbiology*. 2001;**147**(Pt 2):439–47. doi: 10.1099/00221287-147-2-439. [PubMed: 11158361].
40. Klaassen CH, van Haren HA, Horrevorts AM. Molecular fingerprinting of Clostridium difficile isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *J Clin Microbiol*. 2002;**40**(1):101–4. doi: 10.1128/jcm.40.1.101-104.2002. [PubMed: 11773100]. [PubMed Central: PMC120100].
41. Alonso R, Martin A, Pelaez T, Marin M, Rodriguez-Creixems M, Bouza E. An improved protocol for pulsed-field gel electrophoresis typing of Clostridium difficile. *J Med Microbiol*. 2005;**54**(Pt 2):155–7. doi: 10.1099/jmm.0.45808-0. [PubMed: 15673509].