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Phenotypic and Genotypic Characteristics of *Clostridium difficile* Isolates in Patients with Type 2 Diabetes in Iran

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Purpose: This study aimed to investigate the phenotypic and genotypic characterization of *Clostridium difficile* isolates in type 2 diabetes patients with hospital-acquired diarrhea in four teaching hospitals in Isfahan, Iran.

Patients and Methods: A total of 104 hospitalized patients with type 2 diabetes and nosocomial diarrhea were included in the current study over a 2-year period (2015–2017). *C. difficile* isolates were characterized by conventional microbiological methods including the presence of toxin genes, antibiotic resistance testing and molecular methods including multi-locus sequence typing (MLST) and random amplification of polymorphic DNA (RAPD).

Results: All 21 *C. difficile* isolates (20.2%) were detected from 104 studied patients. All isolates were susceptible to metronidazole and vancomycin. The antimicrobial resistance rates were distinctly higher for clindamycin and for moxifloxacin. Based on PCR amplification of *tdA* and *tdB*, 13 isolates (12.5%) carried both of these genes and were considered toxigenic. Thirteen toxigenic *C. difficile* strains were classified into two sequence types (STs), that is, ST54 and ST2 types. The RAPD-PCR amplification patterns of the detected toxigenic *C. difficile* revealed three distinct but related RAPD clusters. RAPD cluster 1 had the highest similarity with RAPD types 2 and 3.

Conclusion: A relatively high rate of CDI was observed in patients with type 2 diabetes and was associated with poorer health outcomes. These patients were exposed to multiple antibiotics and other therapeutic agents. We recommend close screening for the coexistence of CDI and type 2 diabetes in patients with diarrhea using a combination of conventional and molecular methods.

Keywords: *Clostridium difficile*, hospital-acquired diarrhea, RAPD, MLST, molecular characterization, risk factor, type 2 diabetes

Introduction

Clostridium difficile is a strictly anaerobic, gram-positive, sporulating bacillus, identified as an important opportunistic and nosocomial pathogen.^{1,2} *Clostridium difficile* infection (CDI) is the most commonly recognized cause of infectious diarrhea in healthcare settings leading to diseases from the asymptomatic carriage diarrhea to life-threatening colitis.^{1,3,4}

Type 2 diabetes is a common chronic metabolic disease and its prevalence is growing at an alarming rate worldwide.⁵ The American Society of diabetes has estimated that a minimum of 285 million people is affected by type 2 diabetes worldwide, and the prevalence is expected to reach 693 million by the year 2045.^{6,7}

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The numbers for diabetes indicate that over 5 million adults in 2017 (8.9% of the adult Iranian population have diabetes) and it is estimated that by the year 2030, 9.2 million Iranian individuals will have diabetes.^{6,8} increases the risk of recurrent CDI Diabetes-related hospitalization is a serious challenge to the health care system, a situation which may be further aggravated by nosocomial CDI and recurrent CDI.^{2,9}

Epidemiologic characteristics and molecular typing of CDI cases are important tools for the investigation of hospital outbreaks and understanding the modes of transmission.¹⁰ Considering the increased duration of inpatient hospitalization due to type 2 diabetes-related sequelae. Existing data lack information on CDI status in type 2 diabetes patients in our region. The current study aimed to provide a relatively more precise data that might cast light on the issue from a microbiological standpoint using conventional and molecular approaches.

Materials and Methods

Study Design

The current descriptive cross-sectional study involved 104 hospitalized patients ≥ 18 years of age with type 2 diabetes and nosocomial diarrhea admitted to four teaching hospitals in Isfahan, Iran, between April 2015 to May 2017. All cases had acquired diarrhea during hospitalization.

The patients who had been diagnosed with CDI within the last three months before admission to hospitals were excluded from our study. To prevent over-representing, only the first stool sample from each patient was examined. Stool samples were also screened for *E. coli*, *Salmonella spp*, *Shigella spp*, and *Campylobacter jejuni*.

The diagnosis of type 2 diabetes was based on typical symptoms and a fasting plasma glucose level >126 mg/dl (7.0 mmol/l) or a two-hour post-load glucose level of >200 mg/dl (11.1 mmol/l).^{11,12}

A case *C. difficile* infection (CDI) was defined based on clinical symptoms of diarrhea and a positive toxigenic culture.¹⁰ Data on demographic characteristics, recent (within 4 weeks) hospital admission, recent (within 8 weeks) antibiotic treatment and prior medications use including metformin and insulin were collected for all patients.

C. difficile Culture

Stool samples were examined by inoculation into *C. difficile* moxalactam norfloxacin (CDMN) broth 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 treated stool samples were inoculated onto the CDMN- agar surface supplemented with 7% sheep blood and incubated anaerobically for 48 h at 37°C. Plates were examined for suspect colonies with 2–3 mm in diameter, the characteristics p-cresol odor unique to *C. difficile*, ultraviolet light (365 nm) for yellow fluorescence within 1 hr of removal from the anaerobic atmosphere, Gram stain morphology, and positive reaction to L-proline aminopeptidase test (Prodisk, Remeb, Lenexa, KS, USA)^{16,27}

DNA Extraction

Intact chromosomal DNA was extracted using the modified Pitcher et al, (1989) with slight modification for extraction of large-scale DNA¹³ Briefly, cultures of toxigenic *C. difficile* isolates grown in Brain Heart Infusion (BHI) broth for 18 h were centrifuged and washed cells were treated with lysozyme and suspended in TE (Tris, 10 mM; EDTA, 50 mM; pH 8.0). Guanidium thiocyanate and sarcosyl were added to the mixture for protein denaturation. The DNA was purified by phenol-chloroform-isoamyl alcohol. The precipitate was washed in 70% ethanol, dehydrated and dissolved in deionized water and stored in a -20°C freezer until use.^{13–15}

Molecular Identification

All isolates were confirmed by testing the existence of triose phosphate isomerase (*tpi*) gene and screened for the presence of the genes encoding toxin A, B (*tcdA*, *tcdB*) and binary toxins (*cdtA*, *cdtB*). *C. difficile* ribotype 027 was used as a positive control for molecular and microbiological analysis and *C. perfringens* 450 MTCC (Microbial Type Culture Collection) served as the negative control^{16,17}

The patients were divided into 3 groups: i) *C. difficile* culture-negative, ii) non-toxigenic *C. difficile* carriers, and iii) CDI patients.

Multilocus Sequence Typing (MLST)

MLST was performed on all toxigenic *C. difficile* isolates using the primers and methods as described previously by Griffiths et al.¹⁸ Seven housekeeping genes (*adhA*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi*) were amplified and the amplified products were sent to Bioneer Corporation in South Korea for sequencing. DNA sequences were analysed using the PubMLST database (<http://pubmlst.org/>)

cdifficile) to obtain the allele numbers and sequence types (STs).

RAPD Analysis

The PCR-RAPD assay was optimized to ensure the reproducibility and discriminatory power of the method [22,23]. The PCR amplification for RAPD fingerprinting was carried out with the primers AP3 (5'-TCACGATGCA-3') and AP4 (5'-TCACGCTGCA-3') independently in a low-stringency PCR amplification. Each isolate was tested under the same conditions at least twice with these primers.^{14,19}

The PCR products were separated by electrophoresis and scanned on an Uvi-doc gel documentation system. The software DNA FRAG version 3.03 (Nash, 1991) was used to estimate DNA fragment sizes in the RAPD profiles.^{13,20} GelCompar 6.6 software from Applied Maths was used for the construction of dendrogram based on the unweight pair-group method with averages (UPGMA) to estimate the relationships between the isolates. Only major bands were considered and band intensity was not used as a criterion.¹³

Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MICs) of clindamycin, metronidazole, moxifloxacin, rifampin, vancomycin and fusidic acid was determined using the Etest (bioMérieux, France) with log-phase inocula of 10^6 cfu/mL in tubes containing 5 mL of phosphate-buffered saline. All tests performed on pre-reduced Brucella Blood Agar plates containing vitamin K1 (1 mg/mL), haemin (5 mg/L) and 5% defibrinated sheep blood. The plates were incubated anaerobically with a chemical indicator (Microbiology Anaerostest[®] Strips; MERCK, Germany) at 37°C for 48 h.²¹ Interpretation of the results and determination of the MICs were carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute CLSI 2011 guidelines. In EUCAST there are only breakpoints for Vancomycin and metronidazole and for other antibiotics, only epidemiologic cut-offs are available.

Streptococcus sp. MTCC 689 and Clostridium perfringens MTCC 13124 strains were included in each run as controls.^{21,22} The antimicrobial agents tested were chosen because of the emergence of reduced susceptibility.

Statistical Analysis

Data were expressed as the means \pm standard deviations and a $P < 0.05$ was considered statistically significant. The logistic regression model was used to determine the

clinical factors associated with *C. difficile* infection. First, a univariate logistic regression model was fitted on each clinical factor, 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.

We estimated odds ratios (ORs) and 95% confidence intervals (CIs) for each of the variables using logistic regression models. Data were analysed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 16.0.

Results

A total of 21 *C. difficile* isolates (20.2%) were detected from 104 hospitalized patients with type 2 diabetes based on positive culture and the presence of *tqi* gene. Based on PCR amplification of *tcdA* and *tcdB*, 13 isolates (12.5%) carried both of these genes and were considered toxigenic, while the remaining eight isolates (7.7%) were nontoxigenic (Table 1). *Salmonella spp*, *Shigella spp*, *E. coli*, and *Campylobacter jejuni* were not detected in any of the patient samples tested.

The antibiotic susceptibility patterns of 21 *C. difficile* isolates are displayed in Table 2.

All isolates were susceptible to metronidazole and vancomycin and were inhibited by a low concentration of the antibiotics. The MIC₅₀/MIC₉₀ of metronidazole and vancomycin were 0.25/0.5 $\mu\text{g/mL}$, 0.032/0.032 $\mu\text{g/mL}$, respectively. Approximately 50% (11/21) of *C. difficile* isolates were resistant to clindamycin and 33% (7/21) of the isolates to moxifloxacin (Table 2).

We found no obvious relationship between the resistance and toxin genotypes or CDI ($P = 0.21$).

The use of antibiotic was identified in all patients in the 8 weeks prior to CDI diagnosis (Table 1). The stepwise multivariate logistic regression model revealed in patients with diabetes, a history of heart failure (OR 1.35; 95% CI, 1.0–2.6; $P = 0.04$) and prior hospitalization (OR 3.2; 95% CI, 1.3–8.2; $P = 0.001$) were risk factors for CDI. Our findings suggest that in patients with diabetes metformin treatment seems to have a protective effect against the development of CDI (OR 0.52; 95% CI, 0.32–0.89; $P = 0.02$).

Genotypes of *C. difficile* Isolates

Of the 21 *C. difficile* isolates, 13 (61.9%) tested positive for both *tcdA* and *tcdB* (A^+B). There were no deletions in *tcdC* genes in all toxigenic isolates tested. No isolate examined positive for the binary toxin genes *cdtA* and *cdtB*.

Table 1 Clinical Features of 104 Hospitalized Patients with Type 2 Diabetes in Toxigenic *C. difficile* Strains, Negative *C. difficile* Strains and Non-Toxigenic *C. difficile* Strains Groups Admitted to the 4 University Hospitals, Isfahan, Iran (2015–2017)

Variables	CDI, n (%)	Non-CDI, n (%)		Univariate Analysis P value
	Toxigenic <i>C. difficile</i> Isolates, (tcdA ⁺ tcdB ⁺), n = 13	Negative <i>C. difficile</i> Strains, n = 83	Non Toxigenic Strains (tpi ⁺), n=8	
Age, mean (SD)	50.8 (13.3)	52.3 (14.4)	51.6 (14.3)	0.75
BMI (kg/m ²) mean (SD)	26.8 (4.1)	26.1 (5.1)	27.2 (7.4)	0.87
Male gender	7(53.8)	34(40.9)	3(37.5)	0.08
Laboratory values				
White blood cell(10 ⁹ /L) median (IQR) ^a	12.2(9.1–19.2)	10.8(8.7–18.0)	11.5(8.9–18.7)	0.93
Albumin(g/dL)	2.3(2.0–2.4)	2.4(1.96–2.6)	2.1(1.89–2.3)	0.98
Creatinine(mg/dL)	1.2(0.6–1.40)	0.98(0.5–1.21)	1.1(0.6–1.32)	0.86
Symptom				
Fever	8(61.5)	48(57.8)	4(50)	0.89
Abdominal pain	5(38.4)	33(39.7)	3(37.5)	0.91
Abdominal cramp	3(23.1)	17(20.5)	2(25)	0.61
Complications of diabetes mellitus				
Renal failure	3(23.1)	16(19.3)	0	0.09
Hypertension	7(53.8)	53(62.6)	3(37.5)	0.12
Sepsis	1(7.7)	7 (8.4)	0	0.10
Heart failure	5(38.5)	17(20.5)	1(25.0)	0.008
Infected diabetic foot	2(15.4)	14(16.9)	1 (12.5)	0.11
Respiratory failure	1(7.7)	4(4.8)	0	0.14
Gastroenteritis disease	2(15.4)	19(22.9)	1(12.5)	0.16
Prior hospitalization (within 1 mo ^b)	9(69.2)	23(27.7)	2(25)	<0.001
Medication use within 8 weeks prior to CDI test				
Penicillin	7(53.8)	34(40.1)	3(37.5)	0.08
Ciprofloxacin	5(38.5)	29(34.9)	2(25)	0.10
Carbapenem	3(23.1)	11(13.3)	0	0.005
Cephalosporin	9(69.2)	43(51.8)	2(25)	<0.001
Fluoroquinolone	4(30.8)	21(25.3)	1(12.5)	0.20
Metronidazole	0	6(7.2)	0	0.18
Clindamycin	2(15.4)	15(18.1)	1(12.5)	0.78
Insulin	6(46.1)	32(38.5)	3(37.5)	0.006
Metformin	5(38.5)	57(68.7)	5(62.5)	0.009

Note: ^aInterquartile range. ^bHospitalization within one month prior to the study.

Abbreviations: CDI, *C. difficile* infection; SD, standard deviation.

The 21 toxigenic *C. difficile* isolates were classified into two STs groups including, ST54 type (n= 8, 61.5%, tcdA⁺, tcdB⁺, cdtA⁻, cdtB⁻) and ST2 type (n= 5, 38.4%, tcdA⁺, tcdB⁺, cdtA⁻, cdtB⁻) (Figure 1). There was no significant difference between males and females in the prevalence of ST types (P=0.07).

RAPD analysis of *C. difficile* isolates with the primer AP4 produced more informative banding patterns and generated four different RAPD types. RAPD profiles consisted of three to five amplicons ranging from 365 to 1196 base pairs in length. All studied isolates generated three major

common bands, with the fragment sizes of 365, 400 and 510 base pairs. The most prevalent detected RAPD type was RT1 type with 7 isolates (53.8%) followed by RT-2 (4/13, 30.8%), RT-3 (1/13, 7.7%) and RT- 4 (1/13, 7.7%).

The RAPD-PCR amplification patterns of the detected toxigenic *C. difficile* revealed three distinct but related RAPD clusters. RAPD cluster 1 had 94% similarity with RAPD type 2 and 73% similarity with RAPD type 3. The remaining four isolates occurred in two small groups that showed a lower similarity with the other two clusters (Figure 1, Supplementary file).

Table 2 Antimicrobial Susceptibility Testing of 21 Isolates to 6 Antimicrobial Agents

Agent	MIC ($\mu\text{g/mL}$)			No. (%) Isolates Toxigenic <i>C. difficile</i> (n = 13)			No. (%) Isolates Non Toxigenic Strains (n = 8)			MIC Breakpoints (S/I/R) (S/R)
	Range	50%	90%	S	I	R	S	I	R	
Clindamycin ^a	2–256	16	256	4 (30.8)	2 (15.4)	7 (53.8)	2 (25)	2 (25)	4(50)	$\leq 2/4/8 \geq$
Metronidazole ^b	0.032– 2	0.25	0.5	13 (100)	0	0	8 (100)	0	0	$\geq 2^b$
Rifampicin ^a	0.032–34	0.016	0.016	12 (92.3)	0	1 (7.7)	8(100)	0	0	$\leq 8/16/32 \geq$
Moxifloxacin ^a	3–32	2	> 32	8 (61.5)	0	5 (38.5)	6 (75)	0	2 (25)	$\leq 4/>4^b$
Vancomycin ^b	0.016–1.5	0.032	0.032	13(100)	0	0	8(100)	0	0	$\geq 2^b$
Fusidic acid ^a	0.016–2	0.016	0.016	12 (92.3)	0	1 (7.7)	8 (100)	0	0	$<1/>1^b$

Notes: ^aMIC breakpoints applied were those recommended for anaerobes by the Clinical and Laboratory Standards Institute (CLSI).²² The breakpoints are based on epidemiological cut-off values (ECOFFs). ^bVancomycin and metronidazole MIC breakpoints were recommended by the European Committee on Antimicrobial Susceptibility Testing (<http://www.eucast.org>).²¹

Abbreviations: i, intermediate; s, susceptible; r, resistant.

The reference ribotype 027 formed a unique RAPD type pattern, which was distinctly separate from the other isolates.

Discussion

Type 2 diabetes mellitus often leads to an immunocompromised state, subsequent hospitalization, and an increased risk of CDI.⁵ CDI is the most prevalent cause of hospital-acquired diarrhea and is associated with a high mortality rate among elderly and immunocompromised patients who are

hospitalized.^{3,5} Epidemics of CDI over recent decades and the epidemiology of CDI in patients with type 2 diabetes is well studied in North America and Europe.⁹ However, there is relatively little evidence on those associations in developing countries. The need for surveillance of the international mobility of *C. difficile* strains has been emphasized. Circulating strains have capability to spread regionally and internationally, providing adequate ground for close monitoring of the prevalence and molecular epidemiology of CDI in the region. Due to the inaccessibility of health care facilities for *C. difficile* culture and toxin testing in many hospitals of Iran, awareness of circulating strains and their prevalence has been limited. The current study, to our knowledge, is the first cross-sectional study of patients with type 2 diabetes and hospital-acquired CDI in a developing country, Iran.^{2,12}

The incidence of CDI in our patients was estimated at 12.5%. In two similar studies performed in Israel and Malaysia CDI prevalence were reported 30.8% and 8.8% respectively.^{12,23} None of our isolates carried *cdtA* and *cdtB* binary toxin genes, the presence of which is associated with increased mortality of the patients.^{17,24}

The toxigenic *C. difficile* isolates from the studied hospitals exhibited similar epidemic genotype profiles (ST54, ST2). Most of CDI in previous studies were found to be due to A+B+ strains (ST2, ST54) and A⁻B⁺ strains (ST37, ST15).^{25–27} The main epidemic genotypes described recently in different regions of China were ST54, ST37, ST3, ST2 and ST35, however remarkable variations in molecular epidemiology of *C. difficile* across different countries worldwide have been observed.¹ For example, in Korea and Japan, ST17 and ST2 are the predominant types; however, ribotype 027/ST1 is the most common *C. difficile* strain in the United States and Europe, for most Asian countries, ST1 cases have only been reported sporadically.^{1,24,28}

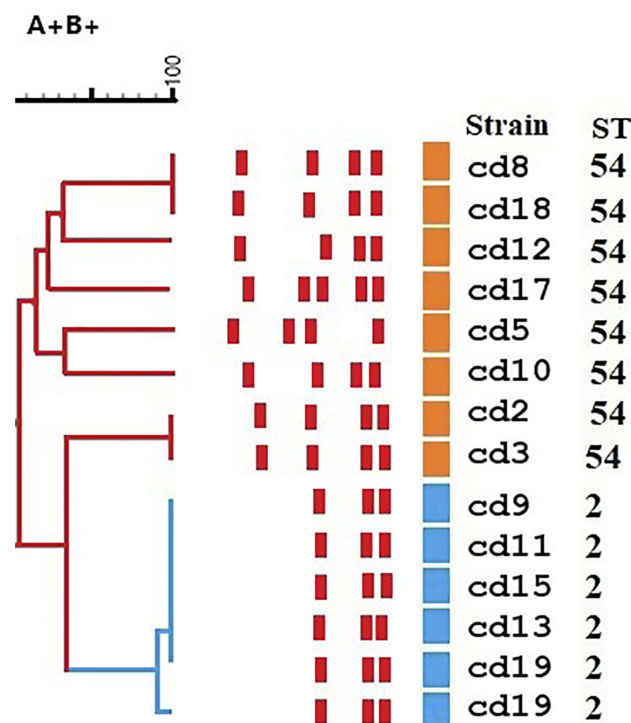


Figure 1 Dendrogram of the detected isolates of *C. difficile*, RAPD-PCR DNA fingerprints with the primer AP4. Dendrogram is color coded according to sequence types (STs) and toxin types.

We used RAPD typing method as a simple, fast and low-cost epidemiologic procedure to find the molecular variation of *C. difficile* isolates. *C. difficile* population structure is clonal and virulence-associated genes exhibited a moderate polymorphism.^{14,19}

RAPD fingerprinting can rapidly classify isolates into clusters and the relationship of strains can be determined.^{13,19} The RAPD typing showed high discriminatory power and stability for analysis of these strains²⁹ This method provide reliable results within one or 2 days and allow for early and more precise implementation of infection control measures.³⁰ Our results strongly suggest that two major RAPD types of *C. difficile*, isolated from 53.8% (7/13) and 30.8% (4/13) of the patients, were prevalent circulating strains in this studied diabetic population. These cases occurred as unique endogenous CDI and might be spread by patient to patient transmission. We combined MLST method as a confirmation step to enhance specificity. Our *C. difficile* isolates were not resistant to metronidazole and vancomycin and in settings where access to vancomycin or fidaxomicin is limited, using metronidazole for an initial episode of nonsevere CDI only is recommended.¹⁰

The rates of resistance to other antimicrobials ranged from 4.8% to 53.8%, which was identical to another study described in Iran, which 5.3% and 89.3% of *C. difficile* strains were resistant to metronidazole and clindamycin respectively.³¹ High-level resistance to these antimicrobial agents can be attributed to different antibiotic regimens used. Antimicrobial use, including many chemotherapeutic agents, was prevalent in both CDI and non-CDI groups, however, the role of antimicrobials in CDI can definitely not be dismissed by these data. The patient age, long length of hospital stay, exposure to antibiotics and immunosuppressant are known main risk factors of CDI.^{2,5,32}

In this study, recent hospitalization and heart failure were risk factors associated with CDI in patients with type 2 diabetes. In agreement with these findings, other studies also identified that severe diseases increase the risk of mortality and morbidity, which is explained by the potential for worsening immune state of these patients.^{5,12}

Metformin treatment has strong effects on the gut microbiome and our findings provide support for the notion that altered gut microbiota could be considered to have a protective effect against the development of CDI.^{23,33}

Conclusion

CDI is not thoroughly studied in Asia and in particular in the Middle East and therefore the extent of diagnosing needs to

be more precisely known. Lack of close monitoring or antibiotic use and unreasonable prescribing in our country indicates that CDI could be widespread in the whole country where proper surveillance is currently absent. The widespread prevalence of strains in Asia indicates that the surveillance for toxin A (*tcdA*) or toxin B (*tcdB*) are more desirable to toxin A assays for diagnosis of CDI. The most virulent strains 027 and 078 have not established in Asia, while ST2 and ST54 types appear to be more prevalent.

In conclusion, our study demonstrated a relatively high CDI rate in patients with type 2 diabetes. The study population was exposed to multiple antibiotics and other therapeutic agents (i.e. metformin, insulin). The use of antibiotics was identified in all patients in the 8 weeks prior to CDI diagnosis and a history of heart failure and prior hospitalization were risk factors for CDI. Metformin treatment seems to have a protective effect against the development of CDI. We recommend close screening for CDI in hospitalized patients with type 2 diabetes using a combination of a rapid genotyping method such as RAPD and a robust high throughput MLST scheme.

Ethics Approval and Consent to Participate

The study was approved by the human research ethics committee at Isfahan University of Medical Sciences and the study was carried out in accordance with the approved guidelines. All the patients provided written informed consent before study entry to their stool being collected and for use in this study. This study was conducted in accordance with the Declaration of Helsinki.

Data Sharing Statement

Data generated or analyzed during this study are available and some are included in this article. (Gel pictures of different PCRs are available).

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Author Contributions

Shoaei P: contributed to acquisition of data and drafting the work and performed microbial and molecular experiments. Shojaei H: Designed and supervised the research, interpreted data, and co-wrote the paper. Shirani K: contributed to the conception of design and revising the draft critically and

collected the patient data. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Disclosure

The authors have no conflicts of interest to declare regarding this paper.

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