

Full Length Research Paper

Arbitrarily primed-polymerase chain reaction (AP-PCR) typing of *Clostridium difficile* isolated from different sources of Imam Reza hospital, Tabriz, Iran

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Detection of the source of *Clostridium difficile* for the control of nosocomial infections produced by these bacteria is very important. For this reason 84 *C. difficile* isolated from 250 stool samples of symptomatic patients, staff, asymptomatic patients at first day of admission, the same patients after seven days of hospitalization and 135 samples from their hospital environment were typed by AP-PCR. In addition to conventional standard tests, gas liquid chromatography was also used as complementary test to identify *C. difficile* isolates. All isolates were separated into 12 genotypes, with 31% falling into group I. Genotypes VIII, X and XII were found only in isolates of symptomatic patients while genotype I was observed in all *C. difficile* of patients and environment. Genotypes III was not detected in any *C. difficile* isolates except in one of the acquired isolates. *C. difficile* is frequently transmitted among hospitalized patients, staff, and their hospital environment.

Key words: *Clostridium difficile*, arbitrarily primed-PCR, gas chromatography.

INTRODUCTION

Clostridium difficile is one of the major cause of nosocomial acquired antibiotic-associated diarrhea and pseudomembranous colitis (Noren, 2010; Conly, 2000) and is an important financial burden on modern hospitals (Conly, 2000). Although it has been established that certain antibiotics, particularly second and third generation cephalosporins increase the risk of infection to *C. difficile* (Conly, 2000), it is important that exposure of hospitalized patients to sources of *C. difficile* is minimized. Numerous *C. difficile* reservoirs exist within hospitals, including contaminated environmental surfaces of different wards (Hota, 2004; Mayfield et al., 2000). Environmental contamination with *C. difficile* spores

occurs as many as 34 to 58% of sites despite cleaning, with surfaces of fomites being most frequently contaminated (Boyce, 2007), normal hospital staff carriage (Kim et al., 1981), colonized new admitted patients (Muto, 2007) and aerial dissemination (Roberts et al., 2008). This huge potential and diverse source provides simple facilities for infection of patients who are under antibiotic therapy. In order to understand the epidemiology of outbreaks and isolated cases, to identify any possible incidence of cross infection, typing of *C. difficile* is necessary. There are several phenotypic and genotypic typing methods (Brazier, 2001). AP-PCR is a simple and suitable technique that has been used

successfully for typing of *C. difficile* in recent years (Killgore and Kato, 1994; Martirosian et al., 1995; Bidet et al., 2000; Rafferty et al., 1998). In this method a single primer approximately 10 nucleotides in length with arbitrary sequence is used. The aim of this study was to compare *C. difficile* isolated from different sources within a general hospital in North West of Iran by AP-PCR.

MATERIALS AND METHODS

Collection of specimens

Eighty-four *C. difficile* isolates recovered from 250 stool samples of symptomatic patients (14/50), staff (18/100), asymptomatic patients at first day of admission (16/50), the same patients after seven days of hospitalization (6 + 16/50) and from 135 samples of their hospital environment (14/135). Ten wards of Imam Reza hospital including, nephrology, gastroenterology, pulmonology, endocrine and rheumatology, neurology, infectious disease, ICU of neurology, ICU of neurology surgery, ICU of general surgery and ICU of pulmonary were chosen based on the length of patients hospitalization. The Imam Reza hospital is situated in Tabriz city with 520 beds in 26 clinical wards, providing services for nearly two thousand patients monthly.

C. difficile were cultured from collected samples by three standard techniques (using Anoxomat: MART Microbiology B.V. the Netherlands 0% O₂, 10% H₂, 10% CO₂, 80% N₂) and suspicious colonies with specific horse odor, characteristic morphology of colonies were identified by conventional biochemical tests as previously described (Akhi et al., 2011).

Gas-liquid chromatography (GLC)

Gas chromatography as complementary test was also used to further identification of *C. difficile* by determination of the volatile short-chain fatty acids (isocaproic acid and isovaleric acid) produced by the growth of *C. difficile* (Johnson et al., 1989; Pepersack et al., 1983). A 48 h growth in thioglycolate broth medium were used to extract volatile fatty acids by adding 0.2 ml of H₂SO₄ (50%) and 1 ml of ethyl ether to 1 ml of the well-mixed culture broth medium. These mixtures were inverted gently several times and centrifuged briefly to separate the phases. One microliter sample of the ether phase was injected into the chromatograph. Volatile fatty acid were separated on a 60 m × 0.25 mm Teknokroma TR CN100 column using a Buck Scientific model 610 gas chromatograph equipped with a split injector and a flame ionization detector. Helium was used as the carrier gas and column linear velocity was set at 20.0 cm/s and the oven temperature at 150°C. The oven temperature program was isothermal for 20 min (Johnson et al., 1989; Pepersack et al., 1983) retention times were identified by injecting known standards (Sigma chemicals).

DNA fingerprinting

Fingerprinting of the 84 *C. difficile* DNA isolates were carried out by arbitrarily primed PCR (AP-PCR). Samples were prepared from overnight growth of *C. difficile* isolates on pre-reduced Columbia agar with 5% sheep blood. DNA of isolates was extracted by sodium dodecyl sulphate-proteinase K modified with N, N, N-trimethyl ammonium bromide (Nikbakht et al., 2008). We chose the ARB11 primer with the sequence of 5'-CTAGGACCGC-3' because it gives clear differences among isolates of *C. difficile* (Fawley and Wilcox, 2001).

Amplification conditions

The total volume of PCR mix was 50 µl including: 10X PCR buffer 5 µl, dNTP mix (10 mM) 1 µl, MgCl₂ (50 mM) 5 µl, primer (10 pmol) 2 µl, Taq DNA polymerase (5 U/µl) 0.2 µl, template DNA 2 µl, sterile redistilled H₂O 34.8 µl. Negative control contained all components except template DNA (Fawley and Wilcox, 2001). The primer and other reagents were prepared according to the manufacturer's recommendations (Cinnagen, Fermentas). PCR reactions were performed with an automated thermal cycler (Eppendorf mastercycler gradient, Germany) with the PCR cycling conditions of initial cycle at 94°C for four minutes, 40 cycles of denaturation at 94°C for one minute, annealing at 30°C for one minute, extension at 72°C for two minutes, and final cycle extension at 72°C for 5 minutes. Amplified DNA was separated by 1.2% agarose gel electrophoresis using Tris-borate EDTA (1x TBE) buffer pH 8 for 2 h at 100 V. Eight microliter of PCR products were mixed with 2 µl of 6x loading buffer (10 mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol and 60 mM EDTA) and filled into the wells. We used 1 Kbp DNA Ladder for estimation of the size of PCR products (Fermentas). After electrophoresis the gels were stained in 0.5 µg/ml of ethidium bromide solution for 15 min. DNA profiles were visualized by means of ultraviolet (UV) light on a UV transilluminator. The gels were photographed using a gel documentation system (UVP, USA) for the analysis of bands. The banding patterns of all lanes were visually compared, and isolates showing identical patterns or variation only in very faint bands were considered the same type.

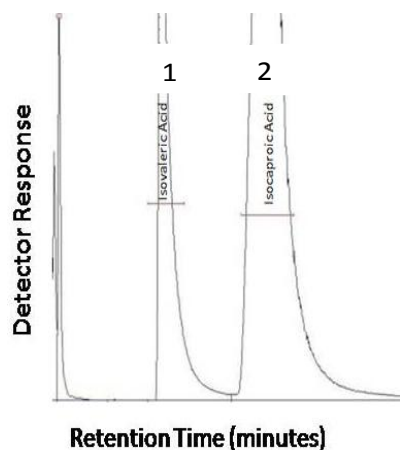
RESULTS

Detection of isocaproic and isovaleric acids has been suggested as a screening test for *C. difficile* in stools (Johnson et al., 1989; Pepersack et al., 1983). Thus, gas chromatographic results for 84 *C. difficile* isolates, already identified by conventional standard tests, were evaluated with respect to isocaproic and isovaleric acids production. Chromatogram for two volatile fatty acids and for standard *C. difficile* strain ATCC 43593 is shown in Figure 1a and b. All 84 *C. difficile* isolated from different sources exhibited this characteristic pattern of peaks, which included isovaleric and isocaproic acids Figure 1c. Amounts of other fatty acids were also present. The non-*C. difficile* clostridia were easily differentiated from *C. difficile*, as they did not produce the same variety or abundance of these fatty acids.

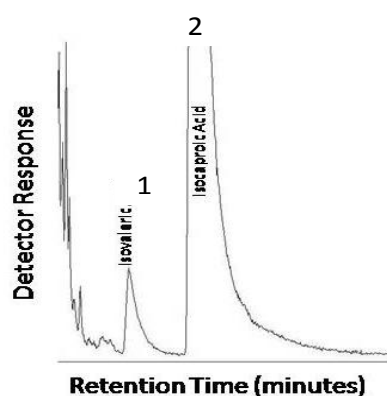
Arbitrarily primed PCR (AP-PCR)

AP-PCR separated the 84 examined isolates into twelve genotypes, numbered I to XII. The frequency of different genotypes among isolates were genotypes I (31%), IV (19.1%), XI (9.5%), V and VII (8.3%), VI and IX (7.1%), VIII (4.8%), XII, X, III and II (1.2%) respectively (Table 1). Genotypes I to XII for *C. difficile* isolated from staff, hospital environments, asymptomatic patients at first day of admission, the same patients after seven days of hospitalization, and symptomatic patients are shown separately in Figure 2.

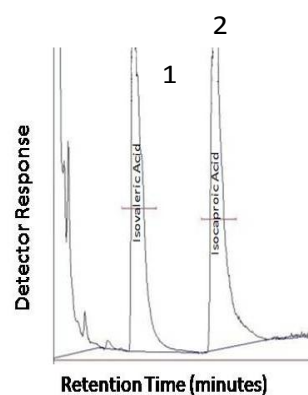
Out of 14 *C. difficile* isolated from environment, three



A



B



C

Figure 1. Chromatogram for standard volatile fatty acids (a), standard strain of *C. difficile* (b) and representative of isolated *C. difficile* (c). 1: Isovaleric acid, 2: Isocaproic acid.

IV (22.2%), V (27.8%), and VI (11.1%) were recognized. Genotype I was the most isolated one among environmental and staff isolates. Five genotypes of I, IV, VI, VII and XI were obtained from 16 isolates of asymptomatic patients at first day of admission. In addition to these genotypes, one more genotype was isolated from the same patients after 7 days of hospitalization (genotype III). Amongst six acquired *C. difficile* after 7 days of hospitalization 3 genotypes of III, IV, and I were observed in which 50% of them was type I. Type IV was mostly imported by newly admitted patients to hospital. Eight genotypes were recovered from 14 *C. difficile* of symptomatic patients in which genotype VIII (28.6%) was the most isolated one and was not obtained from any other *C. difficile* isolates. Genotype I with 1000 bp was the most dominant in all 84 *C. difficile* and exhibited among all samples (Table 1).

DISCUSSION

Outbreaks of nosocomial disease caused by *C. difficile* are relatively common in many hospitals of developed and developing countries (Fawley and Wilcox, 2001; Titov et al., 2000). Laboratory diagnosis of antibiotic associated diarrhea or pseudomembranous colitis requires the isolation of *C. difficile* and the detection of its cytotoxin in the fecal specimen. Laboratory facilities and technical expertise for cell culture are needed, which is also more time consuming. Gas-liquid chromatography is a more rapid and simpler technique and widely is used in bacteriology laboratories to identify anaerobes and to detect their metabolic products directly in clinical specimens. Detection of *C. difficile* by GLC has been reported by microbiology laboratories at different times (Johnson et al., 1989; Pepersack et al., 1983). A high sensitivity (99.6%) and specificity (99%) of GLC in detecting isocaproic and isovaleric acids produced by *C. difficile* in a mixed culture medium has been reported (Johnson et al., 1989) which had a high consistency with the results obtained for identifying of *C. difficile* in our study.

Several typing systems have been described for this microorganism (Brazier, 2001) but AP-PCR has been used by many researches. Williams et al. (1990) showed AP-PCR potentiality as an effective typing system for plants and bacteria. Fawley et al. (2005) used AP-PCR as a tool for typing *C. difficile*. Several scientists to type other organisms (Mahenthalingam et al., 1996) have also carried out this method. In our study all 84 isolates were separated into 12 types which are much higher than the results reported by Titov et al. (2000) (five types) and Fawley et al. (2005) (six types) but lower than the 17 AP-PCR types obtained by Fawley et al. (2005). Sampling from 10 wards with different specialties can be the reason for recovery of 12 types. Acquisition of *C. difficile* during hospitalization varies widely. In our study, 12% of hospitalized patients acquired *C. difficile* after seven days

genotypes I (57.1%), II (7.2%) and IX (35.7%) were detected. Of the 18 isolates of *C. difficile* obtained from staff and typed by AP-PCR, four genotypes of I (38.9%),

Table 1. AP- PCR types of *C. difficile* isolated from patients, staff, and hospital environments.

AP-PCR type	No. of isolates/total no. (%)					Total (%)
	Symptomatic patients	Asymptomatic patients at first day of admission	Asymptomatic patients after seven days of hospitalization	Staff	Hospital environments	
I	2/14 (14.3)	3/16 (18.8)	(**3+3)/22 (27.3)	7/18(38.9)	8/14 (57.1)	26 (31)
II	*NI	NI	NI	NI	1/14 (7.2)	1 (1.2)
III	NI	NI	1/22 (4.5)	NI	NI	1 (1.2)
IV	NI	5/16 (31.2)	(2+5)/22(31.8)	4/18(22.2)	NI	16 (19.1)
V	2/14 (14.3)	NI	NI	5/18(27.8)	NI	7 (8.3)
VI	2/14 (14.3)	1/16 (6.2)	1/22 (4.5)	2/18(11.1)	NI	6 (7.1)
VII	1/14 (7.1)	3/16 (18.8)	3/22 (13.7)	NI	NI	7 (8.3)
VIII	4/14 (28.6)	NI	NI	NI	NI	4 (4.8)
IX	1/14 (7.1)	NI	NI	NI	5/14 (35.7)	6 (7.1)
X	1/14 (7.1)	NI	NI	NI	NI	1 (1.2)
XI	NI	4/16 (25)	4/22 (18.2)	NI	NI	8 (9.5)
XII	1/14(7.1)	NI	NI	NI	NI	1 (1.2)
12	14/14 (100)	16/16 (100)	22/22 (100)	18/18 (100)	14/14 (100)	84/84 (100)

*NI: Not isolated.

**Hospital acquired isolates are shown in bold numbers.

while other researchers reported 16.9 and 9.5% of patients with nosocomial diarrhea had positive infections by *C. difficile* respectively (Rotimi et al., 2002; Sansone et al., 2009). Eleven percent of *C. difficile* acquisition within 72 h after admission to wards was also reported (Samore et al., 1994), considering the period of hospitalization it is much higher than the results we obtained. Patient-to-patient or staff to patient transmission of *C. difficile* was evidenced by incident cases with identical AP-PCR types IV, V, VI, and VII. The presence of these genotypes in the staff and infected patients and absence of those genotypes in the environment over this period implies patient-to-patient and/or staff-to-patient spread.

The dominant type I isolated from all *C. difficile* showed extensive spread of this type among all groups of patients and environment. The type AP- PCR III *C. difficile* 1/22 (4.5%) was isolated from the patients after seven days of hospitalization on only one occasion during the period of study. However, this type was not isolated from samples obtained from environments of wards, staff, asymptomatic patients at first day of admission, and symptomatic patients. Thus, the source of *C. difficile* AP-PCR III isolated from the patient after seven days of hospitalization remains unclear. This may have been introduced by an asymptomatic carrier, via the hands of visitors, or possibly, from an infected patient whose fecal isolate was not available for analysis. Similar to the findings of Fawley et al. (2005) only two types IX and I were identified as causing both patient infection and ward contamination that indicates transmission of *C. difficile* from patient to environment and vice versa. Attempts to determine whether infected patients or contaminated environments are the prime source for cross-infection by

C. difficile had limited success. Some research reported 18 and 10.25% of patients to be stool culture positive at admission to wards, suggesting that *C. difficile*-colonized new admissions are a major source of nosocomial *C. difficile* infection (Clabots et al., 1992; Samore et al., 1994). In our study 32%, of patients admitted to the wards were *C. difficile* positive, among which type XI was imported to the hospital only by admission of new patients. Indiscriminate consumption of antibiotics by patients and people can be the main factor in overgrowth of *C. difficile* among new admitted patients. Types VIII, X and XII were detected only from symptomatic patients and type II was found only in hospital environment.

Conclusion

The results indicate that nosocomial *C. difficile* infection associated with diarrhea is frequently transmitted among hospitalized patients and that the organism is often present on the hospital environment and personnel caring for such patients. Effective preventive measures are needed to reduce nosocomial acquisition of *C. difficile*.

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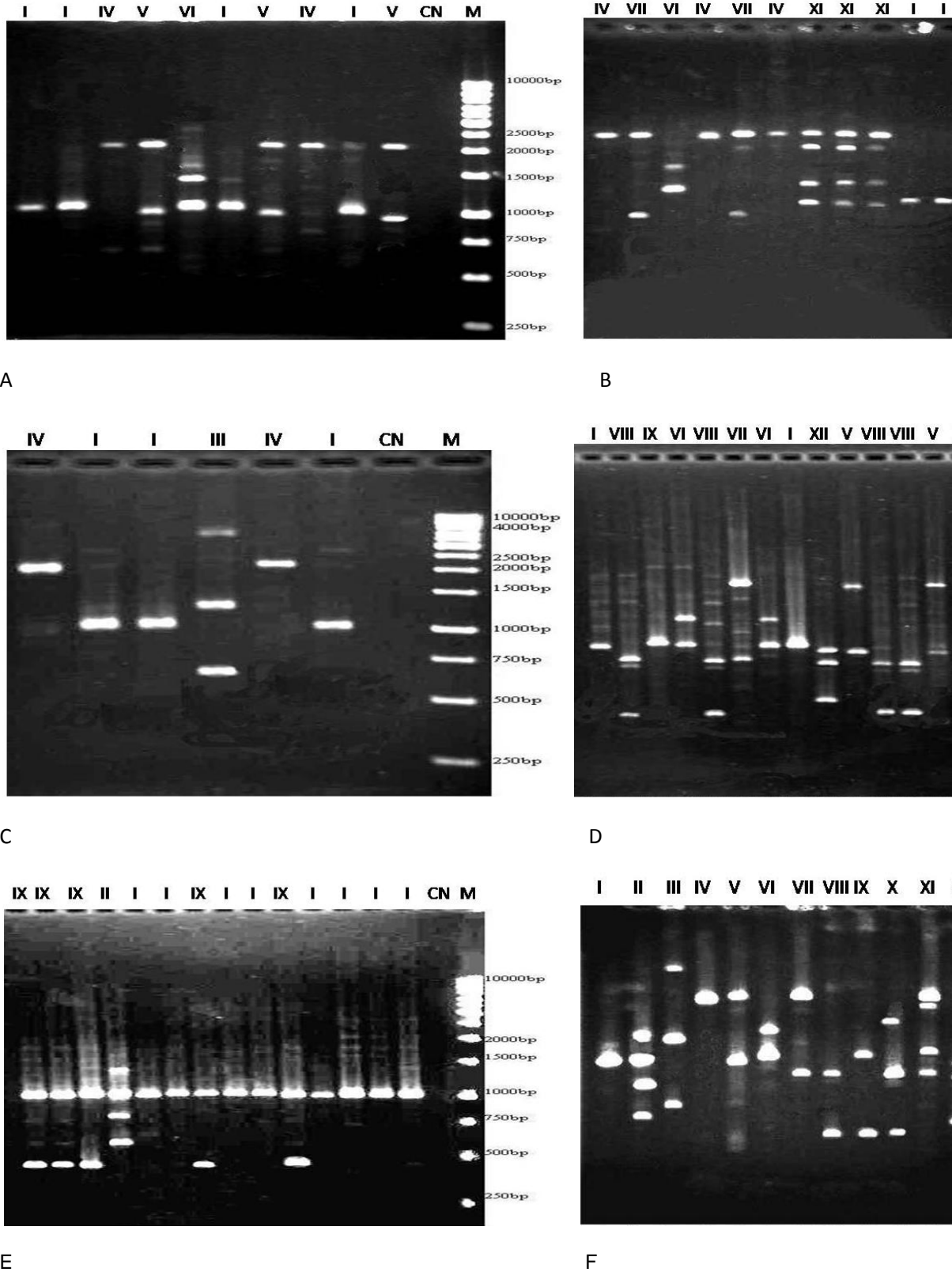


Figure 2. Typing of *C. difficile* isolates from different sources using AP-PCR technique: (a) hospital staff; (b) asymptomatic patients at first day of admission; (c) the same patients after seven days of admission (acquired); (d) symptomatic patients; (e) hospital environment; (f) representative of each genotypes obtained from all 84 isolates. M: 1 Kbp ladder, CN: negative PCR control; I to XII: different genotypes obtained.

research was approved by the committee of ethics on research of the Tabriz University of Medical Sciences (5/4/8265-2009/1/16).

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