Candida Species in Cutaneous Candidiasis Patients in the Guilan Province in Iran; Identified by PCR-RFLP Method

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Abstract- Due to the epidemiological alteration in distribution of *Candida* species as well as significant increasing trend of either intrinsic or acquired in resistance of some of these fungi, the precise identification of *Candida* species is necessary for effective antifungal therapy and also for prevention of nosocomial infections. PCR-RFLP method is indicated to be a reliable, rapid and simple technique which is able to differentiate the *Candida* species. In the present study, we applied this method to evaluate the distribution of *Candida* species in patients affected with cutaneous candidiasis in the Guilan province. 896 clinical cutaneous samples were collected from different parts of skin and nail of suspected patients referred to clinical centers all over the Guilan province during 24 months. Samples were examined directly with 15% KOH and cultured on fungal specific media. Genomic DNA was extracted and the restriction enzyme *Msp1* was applied for polymorphism analysis. Totally, 47 yeast strains were successfully isolated from different clinical samples and identified by conventional as well as PCR-RFLP methods. The results indicated that *Candida albicans* (36.17%) was the most frequent species followed by *C. parapsilosis* (25.53%), *C. tropicalis* (19.14%), *C. guilliermondii* (14.89%), *C. famata* (2.12%) and *C. krusei* (2.12%). Female finger nails were the most common location to be affected by *Candida* species. In conclusion, PCR-RFLP method was successfully used for recognition of clinical *Candida* species within the Guilan province and obtained results revealed *C. albicans* as the predominant causative agent of cutaneous candidiasis. However, distribution of other *Candida* species did not completely consist with the reported distribution of *Candida* species in other parts of Iran with different climate to the Guilan province.

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Introduction

Candida species are able to stimulate opportunistic infections ranged from cutaneous lesions to disseminated mycoses. *Candida albicans* is known to be the major etiologic species of systemic as well as cutaneous candidiasis (1). Recently, the incidence of opportunistic infections caused by other *Candida* species rather than *C. albicans* has progressively increased. In order to manage this epidemiological alteration of *Candida* species, the precise identification of *Candida* species is necessary for effective antifungal therapy (2). In contrast to conventional methods of identification, detection of fungal DNA can be rapid, sensitive and specific by use of PCR-based methods. These methods are being increasingly applied within

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hospital laboratories for the routine detection of pathogenic microorganisms (3). Some modern PCR techniques such as multiplex PCR and multiplex real-time PCR are still require specific and expensive equipment (4). However, the PCR-RFLP is relatively simple and easy to perform. The obtained patterns are recognizable, reproducible and easy to decipher (5-9). As moisture is one of the most important disposing factors for fungal growth, and the Guilan province is located in a high humidity area in the north of Iran, it seems that the incidence of fungal infections particularly candidiasis may increases in this province. In addition, it would be possible that the distribution of candida species in this province is different from the obtained patterns in other parts of Iran. These possible differences could certainly influence on treatment and following up the related infections. Therefore, the precise identification of *Candida* species in this province could help us for effective antifungal therapy and also for controling of such infections in this area. Thus, we benefited the PCR-RFLP method to evaluate the distribution of *Candida* species among patients affected with any kinds of cutaneous candidiasis in the Guilan province.

Materials and Methods

Clinical isolates and morphological identification

From 2010 to 2012 (about 24 months), 896 Clinical samples were collected from different parts of skin and nail of patients referred to 40 laboratories as well as medical centers all over the Guilan province. In order to perform direct examination, portions of cutaneous samples were examined with 15% KOH and also cultured on Sabouraud's dextrose agar supplied with both cycloheximide and chloramphenicol and incubated at 30ºC for 48-72 h. At the early stage of identification, the positive isolates were recognized and by phenotypic methods. Obtained Candida species were then further analyzed and cultured on CHROMagar *Candida* medium (CHROMagar company, France) and incubated at 37ºC for 48h.

Genomic DNA extraction

Fungal genomic DNA was extracted from harvested *Candida* colonies using glass bead disruption method (10). Briefly, about 5 mm^3 of cultured yeast was transferred to a 1.5ml micro-centrifuge tube and 300 mg of 0.5 mm diameter glass beads, 300µl of lysis buffer (100mM Tris-Hcl pH 8, 10 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate), and 300 µl of phenol chloroform-isoamyl alchohol (25:24:1) was added. The samples were shacked vigorously for 5 min, centrifuged for 5 min at 5000 rpm. The supernatant was then transferred to a fresh tube and extracted again with chloroform. High molecular weight DNA was precipitates by adding the same volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The solution was then vortexed and incubated for 10 min at -20°C and centrifuged for 15 min at 12000 rpm. The precipitant was washed with ice-cold 70% ethanol, dried in air, dissolved in 50 µl of double distilled water and stored at -20°C till used for PCR.

Polymerase chain reaction

The reaction was performed with about 50 ng of template DNA in a complete reaction volume of 50 µl containing; 10 µl of 10X PCR buffer, 1mM $MgCl_2$, 0.2 mM of dNTP Mix, 0.5 µM of each primers including: F-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and R-ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and 1.25 U of Taq DNA polymerase. PCR were performed in a thermal cycler (PeQlab, Germany) with the following temperature profile: 1 cycle of 5 min at 94°C, followed by 35 cycles of 30s at 94°C, 45s at 56°C and 1 min at 72°C and a final extension step at 72°C for 10min. Negative controls were included in each test running and the PCR products were stored at -20°C until used.

Restriction digest

The PCR product was digested individually with the enzyme *Msp*I (Fermentas, Germany). Digestion was performed by incubating a 20 µl aliquot of PCR product with 10 U of *Msp*I in a final reaction volume of 30 µl and incubated at 37°C for 2h. Both PCR and RFLP products were then loaded to 2% agarose gels containing 10 mg/ml EtBr (Fermentas, Germany) and analyzed by running for 45 min at 100 V. The observed banding patterns were then used for differentiation of *Candida* species. To discriminate the spices in which *Msp*1 was not able to digest the ITS region, the PCR fragment was subjected for nucleotide sequencing.

Results

Molecular identification

About 258 cutaneous samples were positive for fungal-associated infections at the early stage of identification. Further examinations with 15% KOH as well as culture methods indicated 47 cases of mentioned infections to be caused by members of the genus *Candida*. Afterward, molecular and morphological identification were

performed on obtained yeasts by using PCR-RFLP and CHROMagar *Candida* medium culturing methods, respectively.

Consequently, the internal transcribed spacer regions including; ITS1, 5.8S rDNA, and ITS2, were successfully amplified by PCR analysis in all *Candida* isolates. A single gene fragment in range of 510-870 base pair was obtained in each PCR test. Figure 1a, shows the PCR products of some *Candida* species isolated from different clinical sources. Digestion of amplicons by *Msp*1 restriction enzyme, yielded fragments with size-different banding patterns (Figure 1b). Each pattern was deciphered according to the previously-published standard patterns for *Msp*1 digestion (8). The obtained restriction pattern of each *Candida* species was completely discriminatory, except for *C. famata* which was identified by sequencing afterward. According to obtained digestion patters, *C. albicans* (36.17%) was the most frequent species followed by *C. parapsilosis* (25.53%), *C. tropicalis* (19.14%), *C. guilliermondii* (14.89%), *C. famata* (2.12%), and *C. krusei* (2.12%) (Table 1). Moreover, the distributions of *Candida* species were also evaluated in aspect of gender, age, as well as infected location in studied population. Among 47 species isolated from patients with cutaneous candidiasis, 80.85% of cases were isolated from female. The most affected body location in female was found to be finger nail (30%). However, there were no differences in distribution of candidiasis in various body locations in male (Table 2). Besides, *C. parapsilosis* was reported as the predominant causing agent of toe nail infection in female. Based on the obtained results, the majority of affected cases were in age group of 40 to 50 years old. Table 3 indicates the distribution of *Candida* species in different age groups (Table 3).

Table 1. Distribution of *Candida* species among clinical isolates identified by PCR-RFLP method.

Species	Nr (%)
C. albicans	17 (36/17)
C. parapsilosis	12 (25/53)
C. tropicalis	9 (19/14)
C. guilliermondii	7 (14/89)
C. krusei	1 (2/12)
C. famata	1 (2/12)
Total	47 (100)

Table 2. Distribution of *Candida* species among clinical isolates identified by PCR-RFLP method based on gender and location.

Sex	Species / Location	*C. albicans*	*C. prapsilosis*	*C. tropicalis*	*C. gilliermondii*	*C. krusei*	*C. famata*	Total
Femal	Groin	2	-	-	-	-	-	2 (5.26%)
e	Body	1	-	-	-	-	-	1 (2.63%)
	Toe nail	-	3	1	-	-	-	4 (10.53%)
	Finger nail	11	7	6	5	1	-	30 (78.95%)
	Neck	-	1	-	-	-	-	1 (2.63%)
	Total	14 (82.35)	11 (91.66)	7 (77.77)	5 (71.43)	1	0	38
Male	Foot	-	-	1	-	-	-	1 (11.11%)
	Groin	1	-	-	-	-	-	1 (11.11%)
	Bottom	1	-	-	-	-	-	1 (11.11%)
	Hand	-	-	-	1	-	-	1 (11.11%)
	Finger nail	1	1	1	1	-	1	5 (55.55%)
	Total	3 (17.65%)	1 (8.33%)	2 (22.22%)	2 (28.58%)	0	1	9
Total		17 (36/17%)	12 (25/53%)	9 (19/14%)	7 (14/89%)	1 (2/12%)	1 (2/12%)	47

Table 3. Distribution of *Candida* species among clinical isolates identified by PCR-RFLP method based on age groups.

Age groups	Frequency (%)						Total
	C. albicans	*C. parapsilosis*	*C .tropicalis*	*C. guillermondeii*	*C. krusei*	*C. famata*	
0-10	3	-	-	-	-	-	3 (6.36)
10-20	-	1	1	-	-	-	2 (4.25)
20-30	-	2	-	2	-	-	4 (8.51)
30-40	3	2	2	1	-	-	8 (17.02)
40-50	3	3	4	2	1	-	13 (27.66)
50-60	3	2	-	-	-	-	5 (10.64)
>60	5	2	2	2	-	1	12 (25.53)
Total	17	12	9	7	1	1	47

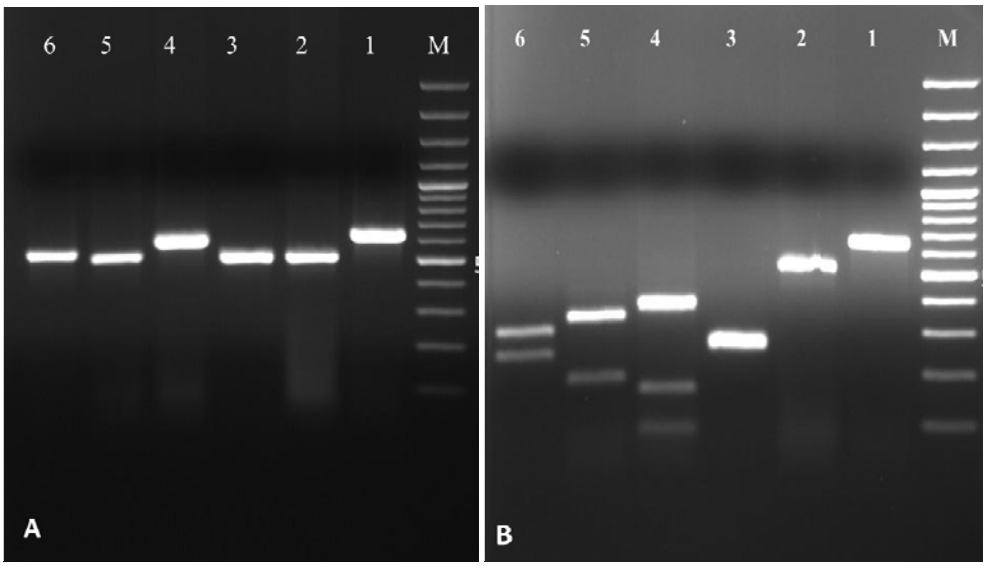

Figure 1. (a) Agarose gel electrophoresis of ITS-PCR products of some of *Candida* species isolated from different cutaneous samples. (b) Restriction digestion of PCR products of *Candida* strain with the *Msp*1enzyme. Line 6: *C. albicans,* Line 5: *C. tropicalis,* Line 4: *C. guilliermondii,* Line 3: *C. krusei,* Line 2: *C. parapsilosis and* Line 1: *C. famata*. Line M: 100 bp molecular size marker.

Appearance of Candida colonies on CHROMagar Candida medium

In order to perform morphological identification, all isolated yeasts were grown on CHROMagar *Candida* medium. After 48 h of incubation, the majority of tested yeast isolates had grown well and formed colonies of 1 to 5 mm in diameter. Our obtained results for morphological identification on CHROMagar *Candida* were approximately consistent with the obtained results of PCR-RFLP. All 17 *C. albicans* isolates formed yellow-green to blue-green consistent with the obtained results of PCR-RFLP. All 17 *C. albicans* isolates formed yellow-green to blue-green colonies on CHROMagar *Candida*. This green color was particularly distinctive of the species. *C. tropicalis* developed a distinctive dark blue-gray color after 48 h of incubation. A dark brown to purple halo in the agar which surrounds the colony was observed as a species-specific colony remark. *C. krusei, C. famata* and *C. parapsilosis* usually do not show specific colony appearance within this medium. However, *C. krusei* colonies were easily distinguishable from two other yeasts with large, rough, spreading colonies having broad, pale edges (data not shown).

Discussion

Precise identification of the yeasts at the species level is essential because of emerging of new pathogen species and increasing in resistance to antifungal agents (11-13). Traditionally, yeasts were categorized based on their morphological, physiological, and biochemical properties (14). However, these conventional laboratory tests are also problematic technically and sometimes hard to decipher. For some fungi such as yeast species, standardized biochemical tests or colorimetric growth media are available (15). Nevertheless, the methods are time-consuming and inaccuracy in the obtained results is possible (8). In contrast to conventional methods, DNA-based approaches are less vulnerable to variations due to growth condition and phenotypic switching. Moreover, they have several advantages over conventional techniques such as speed, accuracy and higher discriminatory power (11). In the present study, we applied PCR-RFLP method in order to differentiate 47 species belonging to the genus *Candida* which have been isolated from cutaneous *Candida*-associated infections. This method has been proved previously to be fast and simple for species recognition (14,16,17). Despite that the incidence of *Candida*-associated infections caused by non-*albicans* species is significantly increased, our result indicated *C. albicans* as the most common causing agent in the Guilan province (36.17%). Compare to the results of the previous studies in other parts of Iran, there was no major changes in the distribution of *C. albicans* in the Guilan province. Shokohi *et al.* applied the same method to identify *Candida* species isolated from cancer patients. Their results indicated *C. albicans* (77%) as the most frequent species (9). Moreover, high frequencies rate were also reported for *C. albicans* in investigations

carried out by Mousavi *et al.* (82%) (18), Mohammadi *et al.* (47%) (18), as well as Zomorodian *et al.* (61%) (19). Although *C. albicans* is mentioned as the most pathogenic *Candida* species in here, however, Compare to the mentioned previous studies, the incidence of this species revealed to be less than 50% of the identified isolates. In addition, according to our obtained results, *C. parapsilosis* was found as the second most common species (25%) while this species has been reported less within patients with candidiasis in other studies. The same difference in reporting of species in the present study was indicated for *C. guilliermondii* (17%) which was near to zero in other previous studies. Besides, there was no isolate of *C. glabrata* in our study. However, almost all previous investigations in other provinces in Iran, have reported different rates of this species. Furthermore, *C. famata* which has been isolated from finger nail of a male patient and reported here (2%) was not isolated from patient with candidiasis in other parts of Iran. This alternation may be due to the source of yeasts (different infection locations) and also due to the climate condition in the Guilan province. The obtained incidence of other candida species isolated in this study was approximately the same as other reports in Iran.

Women seem to be more vulnerable in affecting cutaneous *Candida*-associated infections. Besides, finger nails were found to be the most reported body location to be affected by *Candida* in female (78.95%). This may due to both weather condition and lifestyle in the north of Iran in which the high humidity is considered as a predisposing factor in affecting the disease. Besides, women generally work on farm in mentioned areas, so their hands are constantly exposed to water for a long time. Data showed that elderliness could also be a factor for affecting with cutaneous *Candida*-associated infections.

In conclusion, PCR-RFLP method was successfully used for recognition of clinical *Candida* species within the cutaneous patient in the Guilan province and obtained results revealed *C. albicans* as the predominant causative agent of cutaneous candidiasis.

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