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Identification of *Candida* Species Isolated From Oral Colonization in Iranian HIV-Positive Patients, by PCR-RFLP Method.

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ABSTRACT

Background: The incidence of opportunistic infections due to *Candida albicans* and other *Candida spp*. has been increasing. Rapid identification of candidiasis is important for the clinical management of immunocompromised patients. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a rapid, sensitive, and specific method for detection of clinically important fungi.
Objectives: The purpose of this study was to identify *Candida spp*. isolated from the oral cavities of HIV-infected patients in southeastern Iran (Kerman), by using PCR-based restriction enzyme digestion.
Patients and Methods: We identified 96 *Candida* isolates obtained from 139 Iranian patients infected with the human immunodeficiency virus (HIV), between April 2009 and April 2010, by using PCR-RFLP assay. Universal primers for the internal transcribed spacer (ITS) region (ITS1–ITS4) of the fungal rRNA genes were used for this assay.
Results: We successfully identified the different *Candida spp*. by using the restriction enzyme MspI. *C. albicans* was the most commonly identified species (82.2%), followed by *C. glabrata* (7.29%), *C. parapsilosis* and *C. kefyr* (both 4.1%), and *C. tropicalis* (2%).
Conclusions: PCR-RFLP is a highly sensitive, specific, and direct method for fungal detection and can be used for fungal epidemiological studies in HIV-positive and other immunocompromised patients.

Implication for health policy/practice/research/medical education:
To have knowledge about the HIV positive patients and the role of *Candida* spp. and candidiasis in that group was so important for the authors.

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1. Background

The increased incidences of localized and systemic infections caused by *Candida spp*. during the past decade have been well documented mainly because of the growing numbers of human immunodeficiency virus (HIV)-infected immunocompromised individuals, which are attributable to the HIV pandemic and increased use

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of immunosuppressive therapy in cancer and organ transplant patients. Furthermore, the widespread use of broad-spectrum antibiotics and increased use of invasive procedures (e.g., intubation) and devices (such as drains and catheters) are probably important contributing factors to the incidences of these infections (1-3).

Infections caused by opportunistic agents such as the *Candida spp*. frequently develop in patients with diverse pathological and immunodeficient states such as neutropenia, neoplasia, decompensated diabetes mellitus, malnutrition, organ transplantation, and acquired immunodeficiency syndrome (AIDS) (4). Oral candidiasis develops in 90% of the patients with AIDS, and is the most prevalent opportunistic infection in HIV-infected individuals. In addition, it is an important indicator of disease progression and increased immunosuppression (5). The incidences of opportunistic infections, oral manifestations of HIV infection, and oral candidiasis decreased after the introduction of highly active antiretroviral therapy (HAART) (6).

Traditional methods used for the identification and typing of clinical *Candida* isolates include morphological and biochemical analyses, colony morphotyping, resistogram typing, and serotyping. These techniques are time-consuming and are dependent on phenotypic expression, which makes them potentially unreliable. An alternative method of identification could be genotype-based identification. Genotypic methods have been used extensively for the detection and typing of *Candida* strains, but have been used less frequently for species differentiation (7).

2. Objectives

The aim of this study was to identify the 96 *Candida spp*. that were isolated from the oral cavities of 139 HIV-positive Iranian patients in Kerman between April 2009 to April 2010. We used the internal transcribed spacer (ITS) sequences (ITS1–ITS4) of various *Candida spp*. and the restriction enzyme *Msp*I for identifying *Candida spp*. in this patient group.

3. Patients and Methods

3.1. Sample Collection and Strain Identification

In this study, 139 samples were obtained from the oral cavities of HIV-positive Iranian patients (men, 125; women, 14) with clinically important lesions of oral candidiasis. These patients underwent addiction tests at triangular clinics in a prison in Kerman, between April 2009 and April 2010. All the patients gave written informed consent, and the deputy of the research ethics committee of Kerman University of Medical Sciences approved the study. The oropharyngeal candidiasis (OPC) lesions in HIV-infected patients were diagnosed by an infectious-diseases specialist on the basis of clinical presentation, findings of direct microscopic examination, and positive culture results. OPC lesion samples were obtained from the tongue or the buccal mucosa by using sterile cotton swabs. These swabs were incubated in Sabouraud's dextrose agar with choramphenicol (Merck, Germany) at 32°C for 48 h (under aerobic conditions) and in CHROMagar™ *Candida* (CHROMagar, France) at 35°C for 48 h (in the dark) for production of species-specific colors.

Different chromogenic culture media capable of distinguishing *C. albicans* from other clinically important yeast strains are commercially available. Such media distinguish *Candida* strains from other yeast strains on the basis of the color changes produced by the *Candida* colonies, which are measured using pH indicators and by fermentation of specific compounds or chromogenic substrates for the presumptive identification of *C. albicans*, *C. tropicalis,* and *C. krusei* (8). We used a 10% KOH preparation and Giemsa stain for microscopic examination of pseudohyphae and yeast cell forms. We used carbohydrate assimilation tests with RapID™ Yeast Plus System (Remel, USA) according to the manufacturer's instructions. Fresh yeast colonies were incubated with rabbit serum at 37°C for 3 h to test for germ tube formation. Development of filamentous-form cells and chlamydospore formation were evaluated by culturing the yeast isolates on Dalmau plates (cornmeal-Tween 80 agar) at 30°C for 48 h (9).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed using specific primers for the molecular identification of *Candida spp*. All the samples were incubated in a medium containing 4% glucose, 1% peptone, and 1.5% agar and were incubated at 37°C for 2 days to ensure proper growth.

3.2. DNA Extraction

Genomic DNA was extracted using the glass bead disruption method (10, 11).

3.3. PCR Amplification

The PCR assay was performed using 1 μL of the test sample (about 1 ng) in a final volume of 50 μL. The PCR mix consisted of 10 mM Tris-HCl; master mix 1×; 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM each of dATP, dCTP, dGTP, and dTTP; 0.2 mM each of primers (ITS1: 5′-TCC-GTA-GGT-GAA-CCT-GCG-G-3′ and ITS4: 5′-TCC-TCC-GCT-TAT-TGA-TAT-GC-3′); and 1–2 μL of Taq DNA polymerase. Thirty-five cycles of amplification were performed in a Progene thermal cycler (Techne, England). The initial denaturation was performed at 94°C for 5 min; thereafter, each cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 50°C for 1 min, an extension step at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The amplified products were visualized on 1.5% agarose gel run in tris-borate-EDTA (TBE) buffer (0.09 M Tris, 0.09 M boric acid, and 20 mM EDTA; pH 8.3) and stained with 0.5 μg mL^{-1} ethidium bromide. The stained gel was photographed using the Ultra Violet Photography (Integrated Vision Products®).

3.4. RFLP Assay

The ITS1–ITS4 sequences of various *Candida spp*. were

used in this study. On the basis of the sequences, the restriction sites of different restriction enzymes were determined by the DNASIS software (Hitachi DNASIS® MAX v3.0 Sequence Analysis Software), and the most ideal enzymes were selected. For each restriction digestion reaction, 5 μL of the amplified PCR product was digested with 1.5 μL of restriction enzyme buffer, 0.5 μl (10 U) of the restriction enzyme *Msp*I, and 8 μL of high-performance liquid chromatography—grade water; the reaction mixture (15 μL) was incubated at 37°C for 120 min. Separation of the digested fragments was visualized on 2% agarose gel run in TBE buffer at 100 V for 45 min, and stained with 0.5 μg ml^{-1} ethidium bromide.

3.5. Statistical Analysis

Study data were analyzed using the statistical program STATA (4905 Lakeway Drive College Station, Texas 77845 USA-version 10) for Windows.

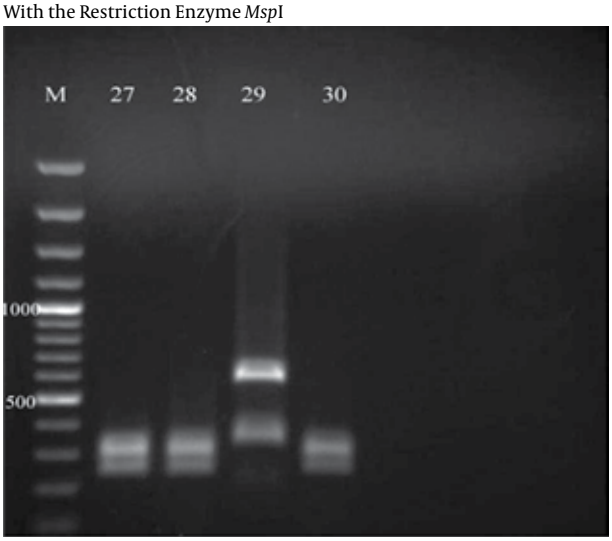
4. Results

Molecular examinations showed oral *Candida* colonization in 69% (96/139) of the patients. *C. albicans* was the most frequently isolated species (82.2%), followed by *C. glabrata* (7.29%), *C. parapsilosis* and *C. kefyr* (both, 4.1%), and *C. tropicalis* (2%). The recognition site for the *Msp*I enzyme is a CCGG sequence (12, 13). The molecular characterization of *Candida spp*. was done on the basis of the number of digested DNA bands in the ITS region. *Candida albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. guilliermondii* and produced 3 bands whereas the others showed 2 distinctive bands after digestion with *Msp*I (10). Size of the pre- and post-digestion ITS1–ITS4 PCR products for *Candida spp*. are reported in *Table 1*. The patterns obtained after *Msp*I restriction digestion of the PCR products of *Candida* isolates are shown in *Figure 1* and *Figure 2*. Table 2 shows the frequency of the isolation of clinically important *Candida spp*. from HIV-positive patients in Kerman, Iran. The data clearly show the predominance of *C. albicans* (82.2%) among the other species. The demographic characteristics of the study population were as follows. Out of a total of 139 HIV-positive patients, 125 were male (89.92%) and 14 were female (10.07%); 61 patients were unmarried (43.88%) and 78 were married (56.11%). The mean of age of the study population was 36 ± 6 years.

Table 1. Size of ITS1–ITS4 PCR Products for *Candida spp*. Before and After Digestion With MspI

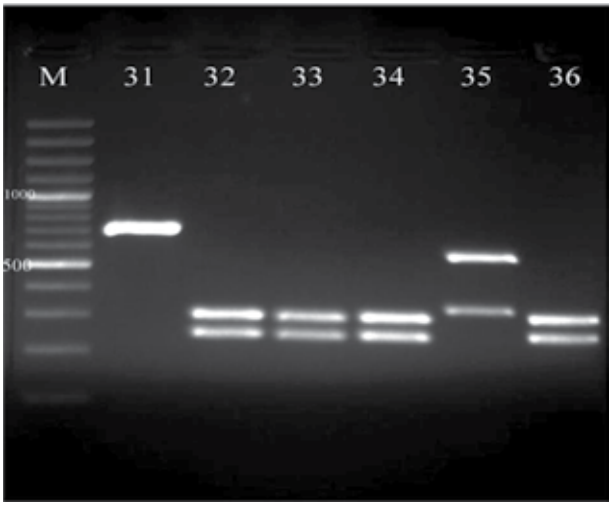
	Size of ITS1–ITS4, bp	Size (s) of Restriction Product (s), bp
C. albicans	535	297, 238
C. glabrata	871	557, 314
C. parapsilosis	520	520
C. krusei	510	261, 249
C. tropicalis	524	340, 184
C. guilliermondii	608	371, 155, 82

Figure 1. Patterns of PCR Products of *Candida* Isolates After Digestion With the Restriction Enzyme *Msp*I



Lane 1: molecular marker (M); lanes 2, 3, and 5 (samples 27, 28, and 30): *Candida albicans;* lane 4 (sample 29): *C. glabrata.*

Figure 2. Patterns of PCR Products of *Candida* Isolates After Digestion With the Restriction Enzyme *Msp*I



Lane 1: molecular marker (M); lane 2 (sample 31): *Candida kefyr*; lanes 3, 4, 5, and 7 (samples 32, 33, 34, and 36): *C. albicans*; lane 6 (sample 35): *C. glabrata.*

Table 2. . Frequency of Isolation of Clinically Important *Candida spp.* From HIV-Positive Patients in Kerman, Iran

	Frequency	
	No	%
C. albicans	79	82.2
C. glabrata	7	7.29
C. parapsilosis	4	4.1
C. kefyr	4	4.1
C. tropicalis	2	2
Total	96	100

5. Discussion

The incidence of opportunistic fungal infections such as candidiasis has considerably increased in recent years. Most of the clinically important fungi belong to the *Candida spp*. (12). Early diagnosis of invasive fungal infections such as candidiasis is necessary to help clinicians administer better treatment decisions and increase the patients' chance of survival. The ability of molecular biology methods to detect fungal pathogens is far superior to that of traditional phenotyping methods (13, 14). Therefore, in the recent past, a variety of molecular biology methods have been applied for the genetic identification of *Candida spp*. Some of these methods include standard PCR, multiplex PCR, PCR with species-specific probes, PCR-RFLP, real–time PCR (15), randomly amplified polymorphic DNA (RAPD)–PCR, DNA sequence analysis, and the mitochondrial large subunit ribosomal RNA (mtLsurRNA)mtLsurRNA gene Sequences (16-18).

In this study, we identified *Candida spp*. by PCR-RFLP method by using 2 universal primers, ITS1 and ITS4, and the restriction enzyme *Msp*I. This method is rapid, easy, and reliable; the method can also be used in clinical laboratories to identify clinically important *Candida spp*. (12). The PCR-RFLP method has been used for the genetic identification of *Candida spp*. in other studies as well (10, 19). Isogai *et al*. and Williams *et al*. used the restriction enzymes *Hae*III and *Hae*III, *Dde*I, and *Bfa*I, respectively, after amplification of the ITS1–ITS4 regions, for the identification of clinically important *Candida spp* (7, 20). *Msp*I does not distinguish between *C. albicans* and *C. dubliniensis*, 2 morphologically similar species of *Candida*. (13, 15).

Our study showed that *C. albicans* (82.2%) was the most frequently isolated species in HIV-positive patients tested in Kerman, Iran. Similar results were observed by Chien-Ching in a Taiwanese population (21) and by Katiraee (9) and Shokohi (13, 14) in Iranian populations. Although *C. albicans* is the most frequently implicated pathogen in OPC, other *Candida spp*. are being increasingly associated with invasive candidiasis (14). The results of our study were different from those shown by Enweani *et al.*, Okungbowa *et al.* and Clark *et al.* (22, 23, 24). In a study on incidence of candidiasis in 103 asymptomatic female students, Enweani *et al.* reported that *C. guilliermondii* was the most commonly isolated pathogen in women who used contraceptive drugs (22). Okungbowa *et al.* reported that the predominant species isolated in the genitourinary tract, in their study, was *C. glabrata* (33.7%), whereas Clark *et al.* (23, 24) reported that the predominant species in cases of bloodstream infection was *C. parapsilosis* (57.8 %, 22/33).

Drug abuse and sexual promiscuity may be important factors influencing the varied distribution frequency of *Candida spp*. across different age-groups and locations (23). Our findings suggest that PCR-RFLP is a simple, useful, and reliable method for identification of *Candida* isolates in mycology laboratories. We showed that *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. kefyr,* and *C. tropicalis* were the major species isolated from HIV-positive patients in southeastern Iran.

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References

1. Lacaz CS, Porto E, Martins JCE, Heins-Vaccari EM, Melo NT. Micologia Médica. 8.ed. *São Paulo: Sarvier*. 2002:123-73.
2. Odds FC. Candida and candidosis. *2nd ed Baillière Tindall, London, England*. 1988.
3. Rippon JW. Medical Mycology. *3rd Edition WB Saunders Co, Philadelphia, USA*. 1988.
4. Lelarge P, Mariot J. Systemic candidiasis [Review]. *Ann Fr Anesth Reanim*. 1992;**11**:558-75.
5. Mesquita RA, Aguiar MCF, Tarquinio SBC, Gomez RS, Bertazzoli RCB. Candidíase oral ea infecção HIV; Oral candidiasis and the HIV infection. *Rev do CROMG*. 1998;**4**(1):27-31.
6. Cassone A, De Bernardis F, Torosantucci A, Tacconelli E, Tumbarello M, Cauda R. In vitro and in vivo anticandidal activity of human immunodeficiency virus protease inhibitors. *J Infect Dis*. 1999;**180**(2):448-53.
7. Williams DW, Wilson MJ, Lewis MA, Potts AJ. Identification of Candida species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol*. 1995;**33**(9):2476-9.
8. Moris D, Melhem M, Martins M, Mendes R. Oral Candida *spp*. colonization in human immunodeficiency virus-infected individuals. *J Venom Anim Toxins*. 2008;**14**(2):224-57.
9. Katiraee F, Khosravi AR, Khalaj V, Hajiabdolbaghi M, Khaksar A, Rasoolinejad M, et al. Oropharyngeal candidiasis and oral yeast colonization in Iranian Human Immunodeficiency Virus positive patients. *J Med Mycol*. 2010;**20**(1):8-14.
10. Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H. A one-enzyme PCR-RFLP assay for identification of six medically important Candida species. *Nihon Ishinkin Gakkai Zasshi*. 2006;**47**(3):225-9.
11. Yamada Y, Makimura K, Merhendi H, Ueda K, Nishiyama Y, Yamaguchi H, et al. Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. *Jpn J Infect Dis*. 2002;**55**(4):122-5.
12. Mirhendi S, Kordbacheh P, Pezeshki M, Khorramizadeh M. Simple and rapid identification of most medically important Candida species by a PCR-restriction enzyme method. *Acta Medica Iranica*. 2003;**41**(2).
13. Shokohi T, Hashemi Soteh M, Pouri ZS, Hedayati M, Mayahi S. Identification of Candida species using PCR-RFLP in cancer patients in Iran. *Indian J of Med Microbiol*. 2010;**28**(2):147.

14. Shokohi T, Bandalizadeh Z, Hedayati MT, Mayahi S. In vitro antifungal susceptibility of Candida species isolated from oropharyngeal lesions of patients with cancer to some antifungal agents. *Jundishapur J Microbiol*. 2011;**4**(Supplement 1):S19-S26.
15. Mirhendi SH, Adin H, Shidfar MR, Kordbacheh P, Hashemi SJ, Moazeni M, et al. Identification of Pathogenic Candida Species: PCR-Fragment Size Polymorphism (PCR-FSP) Method. *TUMJ*. 2008;**66**(9):639-45 [In persian].
16. Campos de Pinho Resende J, Franco GR, Rosa CA, Hahn RC, Hamdam JS. Phenotypic and genotypic identification of Candida *spp*. isolated from hospitalized patients. *Rev Iberoam Micol*. 2004;**21**(1):24-8.
17. Sugita T, Nishikawa A. [Molecular taxonomy and identification of pathogenic fungi based on DNA sequence analysis]. *Nihon Ishinkin Gakkai Zasshi*. 2004;**45**(2):55-8.
18. Yamada Y, Makimura K, Uchida K, Yamaguchi H, Osumi M. Phylogenetic relationships among medically important yeasts based on sequences of mitochondrial large subunit ribosomal RNA gene. *Mycoses*. 2004;**47**(1-2):24-8.
19. Irobi J, Schoofs A, Goossens H. Genetic identification of Candida species in HIV-positive patients using the polymerase chain reaction and restriction fragment length polymorphism analysis of its DNA. *Mol Cell Probes*. 1999;**13**(6):401-6.
20. Isogai H, Mulu A, Diro E, Tekleselassie H, Kassu A, Kimura K, et al. Identification of Candida species from human immunodeficiency virus-infected patients in Ethiopia by combination of CHROMagar, tobacco agar and PCR of amplified internally transcribed rRNA spacer region. *J Appl Res*. 2010;**10**(1):1-8.
21. Hung CC, Yang YL, Lauderdale TL, McDonald LC, Hsiao CF, Cheng HH, et al. Colonization of human immunodeficiency virus-infected outpatients in Taiwan with Candida species. *J Clin Microbiol*. 2005;**43**(4):1600-3.
22. Enweani IB, Ogbonna CI, Kozak W. The incidence of candidiasis amongst the asymptomatic female students of the University of Jos, Nigeria. *Mycopathologia*. 1987;**99**(3):135-41.
23. Okungbowa FI, Isikhuemen O, Dede APO. The distribution frequency of Candida species in the genitourinary tract among symptomatic individuals in Nigerian cities. *Revista iberoamericana de micología*. 2003;**20**(2):60-3.
24. Clark TA, Slavinski SA, Morgan J, Lott T, Arthington-Skaggs BA, Brandt ME, et al. Epidemiologic and molecular characterization of an outbreak of Candida parapsilosis bloodstream infections in a community hospital. *J Clin Microbiol*. 2004;**42**(10):4468.