

Use of restriction fragment length polymorphism to identify *Candida* species, related to onychomycosis

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Abstract

Background: Onychomycosis is one of the most common clinical forms of fungal infections due to both filamentous fungi and yeasts. The genus of *Candida* is one of the most prominent causes of onychomycosis in all around the world. Although *Candida albicans* is still the most frequent cause of nail infections, use of broad-spectrum antifungal agents has led to a shift in the etiology of *C. albicans* to non-*albicans* species. The aim of the present study is rapid and precise identification of *Candida* species isolated from nail infection by using of PCR-RFLP technique.

Materials and Methods: A total of 360 clinical yeast strains were collected from nail infections in Iran. Genomic DNA was extracted using FTA[®] cards. ITS1-5.8SrDNA-ITS2 region was amplified using universal primers and subsequently products were digested with the restriction enzyme *MspI*. For identification of newly described species (*C. parapsilosis* complex), the *SADH* gene was amplified, followed by digestion with *Nla* III restriction enzyme.

Results: *Candida albicans* was the most commonly isolated species (41.1%), followed by *C. parapsilosis* (21.4%), *C. tropicalis* (12.8%), *C. kefyr* (9.4%), *C. krusei* (5.5%), *C. orthopsilosis* (4.1%), *C. glabrata* (2.8%), *C. guilliermondii* (1.4%), *C. rugosa* (0.8%), and *C. lusitaniae* (0.5%). Patients in the age groups of 51-60 and 81-90 years had the highest and lowest distribution of positive specimens, respectively.

Conclusion: Rapid and precise identification of *Candida* species from clinical specimens lead to appropriate therapeutic plans.

Key Words: *Candida* species, onychomycosis, PCR-RFLP

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INTRODUCTION

Onychomycosis encloses all fungal infections of the nail due to filamentous and yeast fungi, accounted

for up to 50% of all nail disorders which ranging from distal and lateral subungual, superficial, proximal subungual and dystrophic onychomycosis; however, in immunocompromised hosts can present a more significant health problem.^[1,2] Studies have shown that the prevalence of onychomycosis increases with age and reasons for the age-related raise in onychomycosis may contain deficient circumferential circulation, repeated nail trauma, longer exposure to pathogenic fungi, diabetes, dysfunction of immune system, incompetence to cut the toenails or keep good foot considerations.^[3,4] Moreover, numerous factors exist for increasing the prevalence of onychomycosis in modern life such as wearing of

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shoes especially high-heeled shoes, high moisture areas (gymnasium and wrestling mattresses by great numbers of people), use of broad-spectrum antibiotics, and corticosteroid therapy.^[6] Although dermatophyte species cause 90% of toenails onychomycosis, *Candida* specie is considered as one of the most important causes of fingernail onychomycosis.^[6,7] *Candida albicans* is the most prevalent species that can cause *Candida* nail infections and invades the entire nail plate, but onycholysis and paronychia may occur.^[7] In cases of the nail *Candida* infections, accurate identification of causative agents at the species level is crucially important due to rapid development of resistance of some species to antifungal drugs, for instance *C. krusei* is innately resistant to fluconazole, and *C. glabrata* is able to be resistant.^[8] Therefore, due to the high degree of phenotypic similarity between *Candida* species, identification problems are imminent. Conventional approaches for identification down to the species level in the diagnostic laboratory are based on morphological and physiological criteria, need several days or weeks to be concluded, and are frequently unspecific. However, today molecular identification methods are well established, e.g., restriction fragment length polymorphisms (RFLP), RFLP with hybridization probes, randomly amplified polymorphic DNA (RAPD) analysis, electrophoretic karyotyping, and sequencing. The latter method is relatively expensive,^[9] therefore, alternative molecular tools with sufficient specificity, reproducibility and sensitivity are necessary.^[9,10] Thus, in the present study, we used polymerase chain reaction (PCR) to amplify the entire internal transcribed spacer (ITS)-rDNA region and secondary alcohol dehydrogenase (SADH) gene, followed by RFLP analysis for identification of *Candida* species were obtained from onychomycosis.

MATERIALS AND METHODS

Fungal strains

A total of 360 clinical yeast strains were obtained from nail infections of patients at Tehran, Isfahan, Alborz, Kashan, and Mazandaran provinces of Iran, from April 2009 to June 2011. All strains were cultured on sabouraud glucose agar (Difco, Detroit, MI, USA) and incubated at 28-30°C for 48 h. A loopful of single and fresh colonies of each strain was transferred to glycerol 20% and stored at -20°C prior to use.

DNA extraction and amplification of the ITS rDNA regions

Genomic DNA was extracted using FTA[®] Elute Micro Cards (Whatman Inc., Clifton, NJ, USA) according to the manufacturer's instructions.^[11] Briefly, fresh colony was suspended in 100 µl of sterile water, then 4 µl of the suspension was dribbled on a disc of FTA card (3 mm in diameter), subsequently incubated at

room temperature for at least 3 hours. The dried papers were eluted in 500 µl distilled water for a few seconds. FTA card was transferred to a new tube containing 30 µl distilled water and incubated at 95°C for 25 minutes. At last the paper discs were taken away and the water containing DNA was kept at -20°C. The internal transcribed spacer region (ITS1-5.8SrDNA-ITS2) region was amplified by using two universal ITS1 (5'-TCCGTAGGTGAACCTGCG G-3') and ITS4 (5'-TCCTCC GCTTATTGATATGC-3') primers.^[12] PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) in 25 µl volumes containing 25 ng of template DNA, 2.5 µl reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM MgCl₂, 0.1% gelatine, 1% Triton X-100), 0.1 mM of each dNTP and 1.0 U Taq DNA polymerase. Amplification was performed with cycles of 2 min at 94°C for primary denaturation, followed by 35 cycles at 95°C (30 s), 55°C (45 s) and 72°C (60 s), with a final 7 min extension step at 72°C.

Digestion with the restriction enzyme *MspI* and *NlaIII*

PCR products were digested in a final reaction volume of 15 µl containing 3 µl water, 1.5 µl buffer, 1U of restriction enzyme *MspI* (Fermentas, Vilnius, Lithuania)^[13] and 10 µl PCR product at 37°C for 2 hours. For identification of newly described species in the *C. parapsilosis* complex including *C. parapsilosis*, *C. orthopsilosis*, and *C. methapsilosis*, the SADH gene was amplified using the SADHF (5'-GTT GAT GCT GTT GGA TTG T-3') and SADHR (5'-CAA TGC CAA ATC TCC CAA-3') primers,^[14] followed by digestion with *NlaIII* (*HinIII*) restriction enzyme (Fermentas, Vilnius, Lithuania).^[15,16] Finally, 5 µl of PCR amplicons and 10 µl of RFLP products were loaded on 1.5 and 2% agarose gel, respectively, in TBE buffer (Tris 0.09 M, Boric acid 0.09 M, EDTA 2 mM) and stained with 0.5 µg/ml ethidium bromide. The bands patterns were visualized using the gel documentation system (UVITEC, Cambridge, UK) and photographed.

Culture on CHROMagar *Candida*

A total of 80 isolates containing *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata* (n = 20 for each species) were sub-cultured on CHROMagar *Candida* (CHROMagar Microbiology, Paris, France), and incubated at 35° C for 2 days observing for color differences for each species^[17,18] for confirmation and the reliability of the PCR-RFLP method.

RESULTS

Since April 2009 to June 2011, 360 clinical isolates were obtained from patients in different geographical locations. According to the size of ITS rDNA region [Figure 1] and *MspI*-RFLP fragments of

strains [Figure 2], the isolates were identified. A total of 77 *C. parapsilosis* complex were screened by amplification of the SADH gene and digested with *Nla*III restriction enzyme [Figure 3]. In this stage, 15 isolates of *C. orthopsilosis* were identified. The most prevalent species was *C. albicans* (41.1%), followed by *C. parapsilosis* (21.4%), *C. tropicalis* (12.8%), *C. kefyr* (9.4%), *C. krusei* (5.5%), *C. orthopsilosis* (4.1%), *C. glabrata* (2.8%), *C. guilliermondii* (1.4%), *C. rugosa* (0.8%), and *C. lusitaniae* (0.5%) [Table 1]. Sixty-nine (19.2%) patients were males and 291 (80.1%) were females. The age range of patients was between 7 and 88 years. The majority numbers of patients were between 51 and 60 years [Table 2]. Sixteen (4.4%) strains were obtained from toenails, and 344 (95.5%) strains from fingernails. *C. tropicalis*, *C. guilliermondii*, and *C. lusitaniae* strains were not isolated from male patients. The results of the identification based on species level observed on CHROMagar *Candida* for 80 selected *Candida* isolates was 100% in agreement with the species delineation in PCR-RFLP method utilized in the present study (data not shown).

DISCUSSION

“Onychomycosis” conventionally referred to non-dermatophytic infection of the nail; however, it is nowadays applied as a common term to designate any fungal nail infection.^[19] *Candida* nail infections occur in patients with chronic mucocutaneous candidiasis which are caused by *C. albicans* and non-*albicans* species. *Candida* onychomycosis occur more frequently in female rather than male and often affect the middle finger which probably due to contact with *Candida* strains that are in the intestine or vagina.^[17,20] Laboratory diagnosis of onychomycosis consists of direct microscopic examination to see fungal elements in nail specimens and culture to identify the species involved. In addition, conventional approaches for identification down to the species level in the diagnostic laboratory are based on morphological and physiological criteria, need several days or weeks to be concluded, and are frequently unspecific. Therefore, discrimination between isolated organisms as pathogens, saprophytes, colonizers, and contamination of the culture plate is a big challenge. Owing to the fact that typically the treatment phase of the onychomycosis is over a long period and takes much time for the nail to grow completely. furthermore non-*albicans* species like *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. krusei* have appeared as significant opportunistic pathogens^[21] and show dissimilarities in levels of resistance to antifungal agents,^[8,22] use of molecular approach

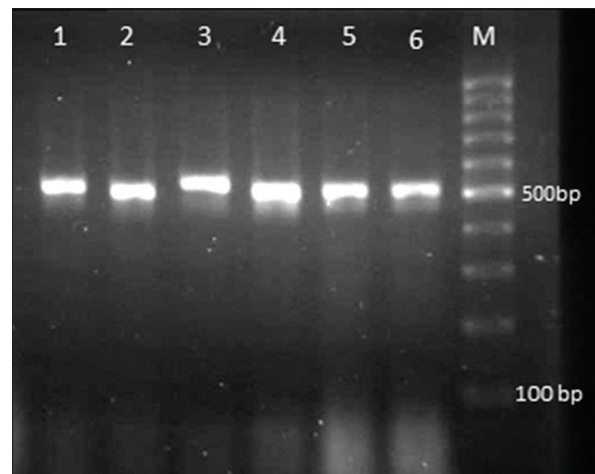


Figure 1: Gel electrophoresis of ITS-PCR products of clinical *Candida* species. Lane 1: *C. tropicalis*, Lane 2,4: *C. krusei*, Lane 3: *C. albicans*, Lane 5,6: *C. parapsilosis*

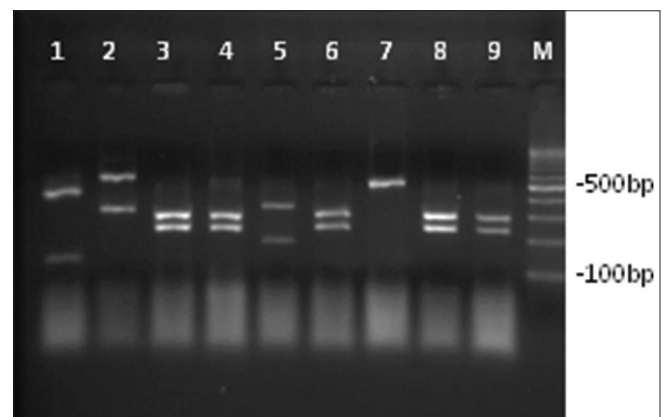


Figure 2: Agarose gel electrophoresis of ITS-PCR products of clinical yeast strains after digestion with *Msp* I. Lane 1: *C. guilliermondii*, lane 2: *C. glabrata*, lane 3,4,6,8,9: *C. albicans*, lane 5: *C. tropicalis*, lane 7: *C. parapsilosis*, and lane M: 100 bp Ladder

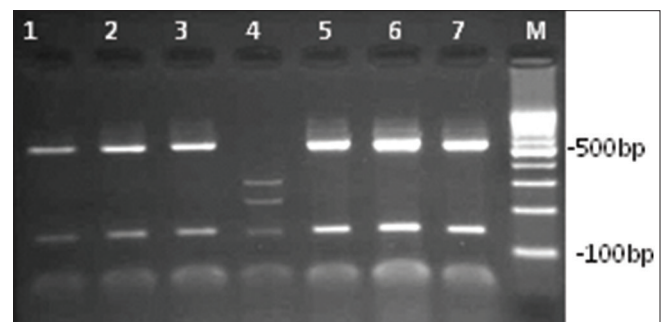


Figure 3: Electrophoretic patterns of SADH-RFLP by *Nla*III restriction enzyme in *C. parapsilosis* group, isolated from onychomycosis in Iran. Lane 1,2,3,5,6,7: *C. parapsilosis*, lane 4: *C. orthopsilosis*, and lane M: 100 bp Ladder

to correctly identify the causative agent based on species level for appropriate antifungal regimen is crucially mandatory. Our study has documented that *C. albicans* caused (41.1%) of onychomycosis and non-*albicans* species connected with (58.9%) nail

Table 1: *Candida* species isolated from nail infections in Iran, from April 2009 to June 2011

<i>Candida</i> species	n (%)	Number of male to female
<i>C. albicans</i>	148 (41.1)	42/106
<i>C. parapsilosis</i>	77 (21.4)	3/74
<i>C. tropicalis</i>	46 (12.8)	0/46
<i>C. kefyr</i>	34 (9.4)	12/22
<i>C. krusei</i>	20 (5.5)	8/12
<i>C. orthopsilosis</i>	15 (4.1)	2/13
<i>C. glabrata</i>	10 (2.8)	1/9
<i>C. guilliermondii</i>	5 (1.4)	0/5
<i>C. rugosa</i>	3 (0.8)	1/2
<i>C. lusitanae</i>	2 (0.5)	0/2

Table 2: Age bracket of patients with onychomycosis in Iran during 2009-2011

Age bracket	n (%)	<i>Candida</i> species
[0-10]	18 (5)	<i>C. albicans</i> (n=6), <i>C. parapsilosis</i> (n=5), <i>C. kefyr</i> (n=4), <i>C. krusei</i> (n=2), <i>C. orthopsilosis</i> (n=1)
[11-20]	27 (7.5)	<i>C. albicans</i> (n=15), <i>C. kefyr</i> (n=5), <i>C. parapsilosis</i> (n=4), <i>orthopsilosis</i> (n=2), <i>C. tropicalis</i> (n=1)
[21-30]	65 (18)	<i>C. albicans</i> (n=31), <i>C. parapsilosis</i> (n=15), <i>C. tropicalis</i> (n=6), <i>C. orthopsilosis</i> (n=4), <i>C. kefyr</i> (n=3), <i>C. guilliermondii</i> (n=2), <i>C. glabrata</i> (n=2), <i>C. krusei</i> (n=2)
[31-40]	61 (16.9)	<i>C. albicans</i> (n=26), <i>C. parapsilosis</i> (n=11), <i>C. tropicalis</i> (n=11), <i>C. kefyr</i> (n=6), <i>C. glabrata</i> (n=4), <i>C. krusei</i> (n=2), <i>C. rugosa</i> (n=1)
[41-50]	50 (13.9)	<i>C. parapsilosis</i> (n=15), <i>C. albicans</i> (n=14), <i>C. kefyr</i> (n=7), <i>C. tropicalis</i> (n=6), <i>C. guilliermondii</i> (n=2), <i>C. glabrata</i> (n=2), <i>C. krusei</i> (n=1), <i>C. rugosa</i> (n=1), <i>C. lusitanae</i> (n=1), <i>C. orthopsilosis</i> (n=1)
[51-60]	70 (19.4)	<i>C. albicans</i> (n=24), <i>C. parapsilosis</i> (n=16), <i>C. tropicalis</i> (n=14), <i>C. kefyr</i> (n=5), <i>C. krusei</i> (n=4), <i>C. orthopsilosis</i> (n=4), <i>C. rugosa</i> (n=1), <i>C. lusitanae</i> (n=1), <i>C. glabrata</i> (n=1)
[61-70]	49 (13.6)	<i>C. albicans</i> (n=22), <i>C. parapsilosis</i> (n=9), <i>C. tropicalis</i> (n=8), <i>C. krusei</i> (n=6), <i>C. orthopsilosis</i> (n=2), <i>C. kefyr</i> (n=1), <i>C. guilliermondii</i> (n=1)
[71-80]	17 (4.7)	<i>C. albicans</i> (n=9), <i>C. parapsilosis</i> (n=2), <i>C. kefyr</i> (n=2), <i>C. krusei</i> (n=2), <i>C. glabrata</i> (n=1), <i>C. orthopsilosis</i> (n=1)
[81-90]	3 (0.8)	<i>C. albicans</i> (n=1), <i>C. kefyr</i> (n=1), <i>C. krusei</i> (n=1)

infections. Several studies have shown an increasing prevalence of onychomycosis with age,^[21,23] but our study did not reveal any meaningful association between these parameters [Table 2]. Tainwala *et al.*^[24] reported only one case of onychomycosis below 20 years, whereas we found 45 (12.5%) positive specimens in age range 0-20 years. Many investigations showed that onychomycosis was more common in males than females,^[25,26] however, the ratio of male to female in our study was approximately 1:4. In the present survey, all fingernail infection caused by *C. albicans*, excluding two cases, had

paronychia which is in conformity with different studies.^[27,28] Amplification and digestion of the ITS rDNA region with *MspI* produced a two-band pattern for *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lusitanae* and *C. rugosa*, in contrast a three-band pattern for *C. guilliermondii*.^[29] Some species like *C. parapsilosis* and *C. kefyr* have no cutting site for restriction enzyme *MspI*; nevertheless, we confirmed our results by amplification of the SADH gene of the *C. parapsilosis* group, following digestion of PCR products with *NlaIII* restriction enzyme.^[15] Since 2005, *C. parapsilosis* complex reclassified to *C. parapsilosis*, *C. orthopsilosis*, and *C. methapsilosis*.^[14] It is considerable that we isolated *C. orthopsilosis* (n = 15) from onychomycosis for the first time in Iran. There was not any *C. methapsilosis* strain among these isolates. *C. kefyr* generated an approximately 720 bp band and were discriminated from the *C. parapsilosis* complex, which presented a 520 bp band. In agreement to our study, Feng *et al.*^[30] reported *C. parapsilosis* was the most prevalent strain of *Candida* onychomycosis among non-*albicans* species. Infections caused by *C. glabrata* are increasing in all clinical forms of candidiasis.^[31] *Candida glabrata* is a complex of species too, comprising two new species *C. nivariensis*^[32] and *C. bracarensis*.^[33] According to the size of variation of ITS-amplicons,^[34] neither *C. bracarensis* nor *C. nivariensis* were identified among our strains. Contrary to our findings, Chai *et al.*^[35] showed that nail infections in consequence of *C. tropicalis* are infrequently seen, whereas we isolated 46 strains from clinical samples. Similar to the current study, Chadeganipour *et al.*^[36] described that *C. albicans* was the most prevalent (84%) species among yeasts, and females were affected more frequently than males. Khosravi *et al.*^[37] reported *Candida albicans* and *C. parapsilosis* as predominant species of the Genus *Candida* that were isolated from onychomycosis. Hashemi *et al.*^[38] revealed 25.5% of onychomycosis cases related to yeast strains and *C. albicans* was the main *candida* species in their study. Rising of non-*albicans* strains among clinical specimens and differences in levels of resistance to the antifungal drugs among *Candida* species are important subjects in medicine. Therefore, molecular techniques are suitable and reliable methods for precise and correct identification of clinical pathogens at the species level which would be useful in clinical laboratories and lead to a perfect control of infections.

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