

**Morphological and Molecular Characteristics
of *Candida pulcherrima*,
an Opportunistic Yeast, Isolated
from Nail Lesions in Iran**

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The aim of this study was PCR-RFLP identification of yeasts that are responsible for onychomycosis. 147 yeast isolates were identified. These yeasts, that had been isolated from fingernails, were identified morphologically at first, and then, for accurate identification, were identified genetically. For this, DNA extraction was performed and ITS region in ribosomal DNA was amplified by PCR. The PCR products were digested by *MSP1* restriction endonuclease. Among these yeasts, *Candida pulcherrima*, a rare non-albicans *Candida* species couldn't be identified by morphological and PCR-RFLP methods, so identification was performed by sequencing of ITS1-5,8-ITS2 segments. This is the first time that *Candida pulcherrima* is isolated from a clinical lesion in Iran. This study showed that for identification of rare non-albicans *Candida* species, we have to use specific methods such as sequencing.

Keywords: Sequencing, Polymerase Chain Reaction, *Candida pulcherrima*.

Introduction

About 50% of nail diseases are caused by fungi and named onychomycosis (1). Some studies have shown that about 30% of fungal infections are onychomycosis (2). Of pathogenic yeasts, those of genus *Candida* are more common. Since they are part of the normal flora of the human mouth, gastrointestinal tract, vagina and skin, they can cause skin, nail and mucosal opportunistic *candida* infections and in the case that patients natural immune system is seriously damaged, these yeasts can cause deep and systemic infections. Different methods are developed to identify the cause of infection, which almost all of them are based on phenotypic characters such as: the morphology of colony and biochemical and physiological tests (sugar absorption and fermentation patterns). Despite their accuracy and validity, these methods are time consuming and are not good enough to identify

some species. New identification methods are based on the study of genotypic characters, like the sequence analysis of certain segments of yeast DNA (3, 4). There are few reports on the isolation of some pathogenic species like *Candida pulcherrima*. In some region, the reports on the isolation of these species have increased when researchers focused on and considered these species. For example in one of these regions during 1971 to 1976, 1720 samples were analyzed on Sabouraud dextrose agar and no case of *Candida pulcherrima* was found. Since 1977, however, the isolation of these yeasts has increased so that during 1977 to 1984 among 4644 analyzed sample, 44 cases of *Candida pulcherrima* were found which 36% of them were of fingernail infections (5). In this paper the importance of the use of PCR-RFLP and sequence analysis of ITS1-5.8-ITS2 segment for the identification of rare *Candida* species has been shown.

Material and methods

147 yeast isolates that had been isolated from onychomycosis lesions were received from Dr. Shidfar's mycology laboratory (Tehran, Iran) and School of Public Health & Institute of Public Health Research- Tehran University of Medical Sciences.

Morphological Tests

Morphological Tests including the appearance and color of colonies on CHROMagar *Candida* medium (biomeriux, France), the production of hyphae or pseudohyphae and chlamydospores in CMA+TW80 medium (Merck, Germany) and the ability of germ tube production in fresh serum were used to identify the yeast species.

Molecular Tests

- 1) DNA extraction

According to previously described method (6), by a bacteriological loop, about 10 mm³ of a fresh colony was transferred to a 1.5 ml eppendorf tube and then 300 µl of lysis buffer (100 mM Tris pH 8, 10 mM pH 8, 100 mM NaCl, 1% SDS, Triton 2% X-100), 300 µl of phenol: chloroform (1:1) and 200 µl of glass beads, with a diameter of 1 mm, were added and the tube was vigorously shaken. Then, the sample was centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to a clean tube and 400 µl of chloroform was added. After centrifuging as the previous conditions, the aqueous phase was transferred to a clean tube and then 1 volume of cold isopropanol and 5 of 3M sodium acetate (pH: 5.2) were added and was kept at -20 °C for 10 minutes. After that, the sample was washed by 70% ethanol. Then 50 µl distilled water was added and the sample was kept at -20 °C

2) Polymerase Chain Reaction (PCR)

PCR was performed to amplify ITS1-5.8S-ITS2 segment in ribosomal DNA. For this, ITS1-5.8S-ITS2 universal primers were used. The sequences of ITS1 and ITS4 were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. The components of PCR reaction were as follows: 2.5 µl of 10x PCR buffer, 1.5mM MgCl₂, 0.5 µl of 10 mM dNTPs, 0.4µM Primers, 1.25 units of Taq polymerase (Sinagene, Iran), 1 µl of template DNA and molecular grade dH₂o up to 25 µl. The temperature cycles were as follows: initial denaturation at 95 °C for 6 min, 30 cycles of 30 sec at 94 °C, 45 sec at 56 °C and 1 min at 72 °C and finally 72°C for 7 min (7).

3) Restriction Fragment Length Polymorphism (RFLP)

After confirming PCR products on agarose gel, they were digested by *MSP1* (Roche Molecular, Germany). The components of digestion reaction were as follows: 10 µl of PCR products, 1.5 µl of digestion buffer, 5 units of *MSP1* enzyme, and dH₂o up to 15 µl. The prepared sample then was placed at 37 °C for 3 hours. Enzymatic digestion of PCR products of different yeasts will produce

different patterns. The digested fragments were then electrophoresed through 1.8% agarose gel and then visualized by ethidium bromide staining (8).

4) ITS sequencing

After amplification of ITS1-5.8S-ITS2 segment, 20 μ l of PCR products were sent to kawsar Biotech Company (Tehran, Iran) for sequencing.

Results

In this study 147 yeast isolates that were of 9 different species of *Candida* genus were identified. 87.1% of these isolates were common species including: *Candida albicans* (45.6%), *Candida parapsilosis* (22.5%), *Candida tropicalis* (21.8%), and also the rare *Candida* species including *Candida glabrata*, *Candida krusei*, *Candida kefir*, *Candida guilliermondi*, *Candida glabrata*, *Candida lusitaniae* and *Candida pulcherrima* were identified. This is the first report of *C. pulcherrima* in Iran. Figures 1 and 2 shows *Candida* species on agarose gel. Figure 3 shows the growth of *C. pulcherrima* colonies on CHROMagar *Candida* medium. Figure 4 shows the sequence of ITS segment of *C. pulcherrima*.

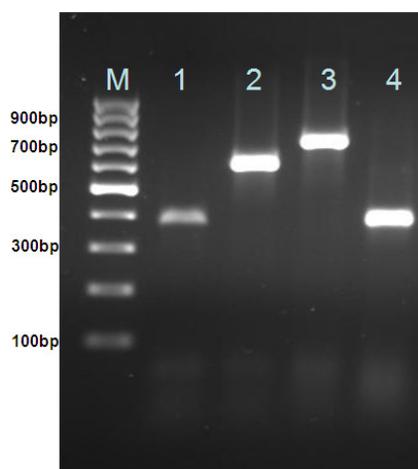


Figure 1. Electrophoresis of PCR products, 1: *C. lusitaniae*, 2: *C. guilliermondi*, 3: *C. kefir*, 4: *C. pulcherrima*.

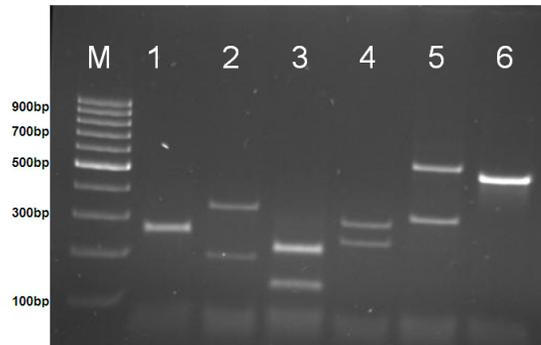


Figure 2. PCR-RFLP profile of samples, 1: *C. krusei*, 2: *C. tropicalis*, 3: *C. pulcherrima*, 4: *C. albicans*, 5: *C. glabrata*, 6: *C. parapsilosis*.



Figure 3. *Candida pulcherrima* colonies in CHROMagar Candida media.

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ACACAATTAAAAAAGTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
CGCAGCGAATTGCGATACGTAATATGACTTGCAGACGTGAATCATTGAATCTTTG
AACGCACATTGCGCCCCGGGGTATTCCCCAGGGCATGCGTGGGTGAGCGATATTT
ACTCTCAAACCTCCGGTTTGGTCTGCTTCGGCCTAATATCAACGGCGCTAGAAT
AAGTTT

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Figure 4. Shows the sequence of ITS segment of *C. pulcherrima*.

Discussion

There are more than 150 yeast species that cause human disease. The identification of pathogenic yeasts is usually based on chemical and morphological tests which are expensive and time consuming. These methods could identify a few species and aren't able to distinguish closely related species. Molecular methods are rapid and sensitive alternatives for the identification of these species. A molecular method that is used and has a high sensitivity is the sequencing of certain segment(s) of chromosomal DNA. By sequencing of ITS segment of rDNA gene cassette, for example, it is possible to identify about 40 clinically important yeast species (9). In this study, we used PCR-RFLP method to identify all isolates. One out of 147 isolates (about 99.4%) were successfully identified by this method. The unidentified isolate was identified by sequencing of ITS1-5.8S-ITS2 segment and it was shown to be *Candida pulcherrima* species. This is the first report of this species in Iran. The color of *Candida pulcherrima* colonies on CHROMagar *Candida* was chrome. The ITS segment of this species has length of 350 bp and the enzymatic digestion of this segment by *MSP1* endonuclease will produce 2 fragments of 130 and 220 bp length. As the figure 2 shows, the enzymatic digestion of this region by *MSP1* enzyme will produce distinct electrophoretical patterns for *Candida* species. Blasting the sequence against Gene Bank shows 100% homology between this segment sequence and *Candida pulcherrima*. There has been a few studies on *Candida pulcherrima*. In a study by Canteros and colleagues 41 non-albicans *Candida* species were identified, of them 6% were *Candida pulcherrima* (10). During 1971 to 1976 among 1720 analyzed samples from skin, nail, hair, and phlegm of patients with skin diseases, Pospisil reported just one case of *Candida pulcherrima*, while he reported 44 cases of this species among 4644 samples which most of them were of nail (5) during 1977 to 1984. This species has been isolate from dog skin lesions by Kozak and colleagues (11). The treatment of onychomycosis is still remained a health problem. The lack of enough knowledge and also the lack of a robust

identification method are probably of the main reasons for this problem. We strongly recommend the use of molecular methods like PCR-RFLP and sequencing of certain regions such as ITS1-5.8S-ITS2 for robust identification of causative fungi in mycology reference laboratories.

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