

Use of cost effective and rapid molecular tools for identification of *Candida* species, opportunistic pathogens

Rezazadeh E¹, Moazeni M^{2,3}, Sabokbar A^{1*}

¹ Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran

² Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

³ Department of Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

*Corresponding author: Azar Sabokbar, Department of Microbiology, Islamic Azad University, Karaj Branch, Karaj, Iran.

Email: sabokbar@kiauo.ac.ir

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Abstract

Background and Purpose: Candidiasis is a widespread fungal infection caused by different *Candida* species. Rapid identification of *Candida* species in clinical laboratory is becoming increasingly important since the identification and discrimination of ethological agents for early treatment. We aimed at molecular identification of commonly *Candida* species isolated from clinical samples by using both PCR-RFLP assay and amplification of hwp1 gene.

Materials and Methods: Clinical samples comprising of vaginal specimens, cutaneous, sputum, bronchoalveolar lavage (BAL), and blood cultures were recovered from suspected patients. *Candida* isolates were initially identified phenotypically and confirmed by molecular approaches based on restriction fragment length polymorphism (PCR-RFLP) with *MspI* restriction enzyme. Amplification of hwp1 gene was performed for discrimination of *C. albicans* from *C. dubliniensis* and *C. africana*.

Results: The most abundant species were *C. albicans* (n=67; 44.6 %), *C. glabrata* (n=10; 20 %), *C. tropicalis* (n=20; 13.3 %), *C. krusei* (n=12; 8 %), *C. parapsilosis* (n=11; 7.3 %). Out of 67 *C. albicans* species, 6 species identified as *C. dubliniensis* and 4 species identified as *C. africana*.

Conclusion: High frequency of non-*albicans* *Candida* species and differences in levels of susceptibility to the antifungal agents are important issues in medicine. Therefore, to manage the *Candida*-related infections properly, molecular diagnostic methods would be fast, reliable and even cost-effective approaches for identification of *Candida* species.

Keywords: *Candida* species, Candidiasis, hwp1, PCR-RFLP

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Introduction

Dandidiasis is a widespread fungal infection caused by different *Candida* species especially in patients infected with human immunodeficiency virus (HIV), immunocompromised individuals, intravenous drug abusers, patients undergoing hematopoietic stem cell and solid organ transplant recipients, and those who present underlying valvular heart diseases or prolonged use of intravenous catheters [1]. Although *Candida albicans* is responsible for up to 78 % of nosocomial candidiasis [2], reports describing cases of *Candida* infections by non-*albicans* *Candida* species have been also documented [3]. Consequently, it is important to identify the causative organism to the species level correctly. Rapid identification of *Candida* species in clinical laboratory is becoming increasingly important since the identification and discrimination of etho-

logical agents for early treatment, and preventing the invasion is highly recommended [4]. Due to the high degree of phenotypic similarity between *Candida* species, identification problems are imminent. Conventional approaches for identification down to the species level are not very effective in detecting yeast [5]. Therefore, Alternative molecular assay with high specificity, reproducibility and sensitivity are necessary [6, 7]. The objective of current study was to identify the common *Candida* species isolated from clinical samples by using both polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and amplification of hwp1 gene.

Materials and Methods

Yeast isolates

Clinical samples comprising of vaginal specimens, cutaneous, sputum, bronchoalveolar

lavage (BAL), and blood cultures were recovered from suspected patients during routine clinical administration at several hospitals of Tehran and Sari, Iran. Initially, obtained specimens were inoculated onto Sabouraud dextrose agar (SDA, Difco) supplemented with chloramphenicol and incubated at 35°C for up to 2 days. The obtained strains were preliminarily identified to the species level based on phenotypic characterizations, i.e., colony color on CHROM agar Candida medium (CHROMagar Company, Paris, France), germ-tube formation, microscopic morphology on corn-meal agar (Difco laboratories, Detroit, Mich., USA) with 1 % tween 80. Identified strains were deposited to the reference culture collection of Invasive Fungi Research Center (IFRC), Sari, Iran.

DNA Extraction

Genomic DNA was extracted, using glass bead disruption. Briefly, 300 µl of lyses buffer (10 mM Tris, 1 mM EDTA (pH= 8), 1 % SDS, 100 mM NaCl, 2 % triton X-100), 300 µl of phenol chloroform (1:1) solution and equal to 300 µl of 0.5 mm diameter glass beads, were added to yeast. After 5 min of vigorous shaking which followed by 5 min centrifugation at 10000 rpm, the supernatant was isolated and transferred to a new tube and equal volume of chloroform was added, mixed gently, centrifuged and its supernatant was transferred to a new tube. For alcohol precipitation, 0.1 mL volume sodium acetate (pH= 5.2) and 2.5 mL volume of cold absolute ethanol were added and the mixture was gently shaken and centrifuged at 10000 rpm for 10 min at 4°C. After washing with 70 % ethanol, the pellets re-suspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA) and were stored at -20°C prior to use.

RCR- RFLP analysis

The PCR-RFLP method was performed as previously described [6]. Briefly, PCR amplification of ITS1-5.8S-ITS2 rDNA regions was achieved using the universal primers ITS1 (5' -TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5' -TCCTCCGCTTATTGATAT GC-3') (MWG-Biotech AG, Germany). PCR amplification was performed in a final volume of 50 µl. Each reaction consists of 2 µl template DNA, 0.5 µl of each primer at 25 pmol, 1.25 µl of dNTP (BIORON GmbH, Germany) at 5 mM, 0.5U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 5 µl 10× PCR buffer. The PCR conditions were as follow: 35cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. Subsequently, PCR

products were digested in a final reaction volume of 15µl containing 3µl water, 1.5µl buffer, 1U of restriction enzyme *MspI* (Fermentas, USA) and 10µl PCR product at 37°C for 2h. Amplified and digested products were visualized by 1.5 % and 2 % agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid and 2 mM EDTA, pH 8.3) respectively, and stained with ethidium bromide (0.5 µg/ml) and photographed.

Partial amplification of the *hwp1* gene

Definitive species identification and discrimination of all members of the *C. albicans* species complex (*C. albicans*, *C. dubliniensis*, *C. africana*, and *C. stellatoidea*) was performed by the amplification of the hyphal wall protein 1 (*hwp1*) gene as described by Romeo and Criseo [8]. PCR amplification of *hwp1* gene was achieved using the forward, 5'-GCTACCACTTCAGAATCATCATC-3' and reverse, 5'-GCACCTTCAGTCGTA GAGACG-3' primer pairs. The partial amplification of the *hwp1* gene was used to distinguish *C. albicans* isolates (941 bp) from atypical form i.e., *C. africana* (750 bp) and *C. dubliniensis* (569bp). However, results were confirmed by sequencing of the ITS rDNA regions using previously described primers [9]. Briefly, sequencing was performed on an ABI 3730xl automatic sequencer (Applied Biosystems, Foster City, CA) and adjusted using the SeqMan of Lasergene software (DNASar Inc., USA.) and compared with GenBank database (<http://www.ncbi.nlm.nih.gov/>).

Results

One hundred and fifty clinical specimens were collected from patients with candidiasis. Clinical samples were obtained from vagina (67.5 %), skin scraping (13.2 %), sputum (8.3 %), BAL (6.8 %), and ear discharge (4.2 %). Figure 1 demonstrates the patterns of ITS-RFLP for *Candida* strains after digestion with *Msp I*. The most prevalent species isolated from patients were *C. albicans* (n=67; 44.6 %), *C. glabrata* (n=30; 20 %), *C. tropicalis* (n= 20; 13.3 %), *C. krusei* (n=12; 8 %), *C. parapsilosis* (n=11; 7.3 %). PCR amplification of *hwp1* gene was used for discrimination of *C. albicans*, *C. dubliniensis*, *C. africana*, and *C. stellatoidea*. Figure 2 shows the patterns of PCR amplification of *hwp1* gene. Out of 67 *C. albicans* species, 6, 4 and were identical as *C. dubliniensis* and *C. africana* respectively. There were no significant differences observed between the distribution and origin of *Candida* isolates. However, *C. africana* and *C. glabrata* were identified most in vaginal samples ($P \leq 0.01$).

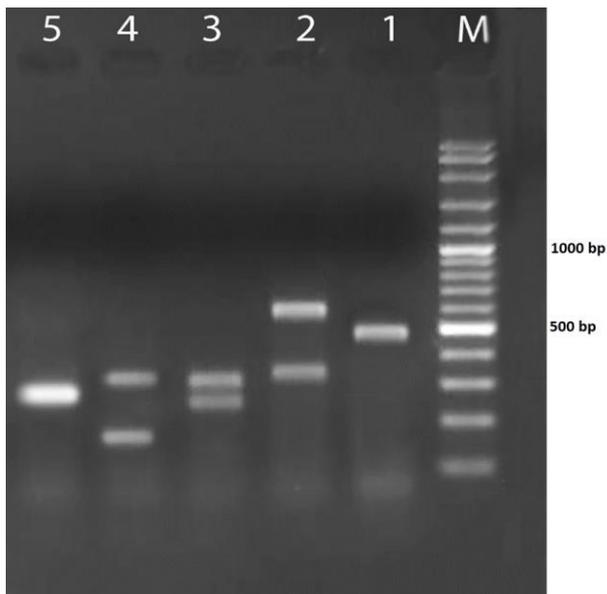


Figure 1. Patterns of PCR-RFLP products of *Candida* isolates after digestion by the restriction enzyme *MspI*; Lanes 1, represent *C. parapsilosis*; 2, *C. glabrata*; 3, *C. albicans*; 4, *C. tropicalis* and 5, *C. krusei*. M: 500 bp DNA marker

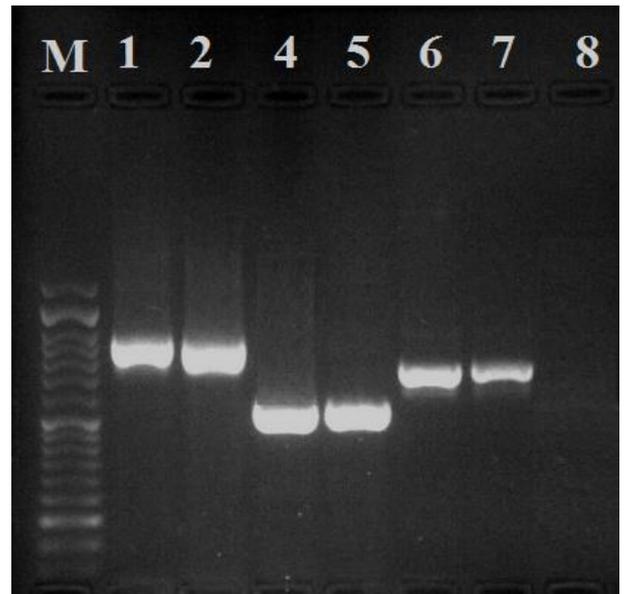


Figure 2. Species-specific amplification of the hwp1 gene; Lane 1- 2 for *C. albicans*; Lane 4-5, for *C. dubliniensis*; Lane 6-7, for *C. africana*; Lane 8, for Negative control. Lane M is 100 bp ladder molecular size markers

Discussion

Recently, the increased incidence of severe candidiasis has been attributed to the enlargement of the population of high risk patients, which is resulted from advances achieved by modern medicine, immunosuppressives, and broad-spectrum antibacterial drugs [10]. Despite the advances in medical interventions and the development of new antifungal agents, the mortality rate remains high [2]. Because of high degree of phenotypic similarity among species of *Candida*, identification problems are imminent [11]. Identification down to the species level based on conventional criteria is time consuming and need expertise. This report provides the species distribution of clinical isolates of *Candida* species with molecular identification. In this study, *C. albicans* was the most common species associated with *Candida* infection (44.6 %), followed by *C. glabrata* (20 %) which is in agreement with other studies [2, 12, 13], however the overall percentage of non-*albicans* species (55.4 %) was higher than in previous reports [13]. Shokohi *et al.* and Papon *et al.* mentioned *C. glabrata* as the most common non-*C. albicans* species in their investigation [14, 15]. However, Alborzi *et al.* showed that the most abundant species isolated from Iranian patients were as follow; *C. albicans* (50 %), *C. glabrata* (21.4 %), *C. dubliniensis* (13.3 %), *C. krusei* (9.8%), *C. kefyr*

(3.1 %), *C. parapsilosis* (1.6 %), and *C. tropicalis* (0.8 %) [16]. These differences in rates might be attributable to differences in the representation of the populations studied and ecological and climatological factors. The present study found that *Candida* species isolated from patients identified as *C. dubliniensis* (n=6) and *C. africana* (n=4). Solimani *et al.*, also showed that 7.1 % of species from *C. albicans* identified as *C. dubliniensis*, however *C. africana* strains were not found [17]. In addition, Yazdanparast *et al.* believe that based on molecular tools, *C. africana* constituted 4.38 % of the vaginal isolates of the *C. albicans* species complex [11]. In this study, we performed PCR-RFLP assay and amplification of hwp1 gene to identify the clinically important *Candida* species and we found that *C. albicans* as predominant species isolated from this clinical samples [6, 17, 18].

Conclusion

High frequency of non *albicans Candida* species and differences in levels of susceptibility to the antifungal agents are important issues in medicine. Therefore, to manage the *Candida*-related infections properly, molecular diagnostic methods would be fast, reliable and even cost-effective approaches for identification of *Candida* species.

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Authors' contributions

A.S. designed and managed the research. E.R. performed the tests. M.M. wrote the draft and edited the final manuscript.

Conflicts of interest

No potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Financial disclosure

No financial interests related to the material of this manuscript have been declared.

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