



Molecular Identification of Clinically Common and Uncommon Yeast Species

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Received 2018 January 13; Revised 2018 August 22; Accepted 2018 September 03.

Abstract

Background: The incidence of fungal infections caused by the yeasts and yeast-like species increased dramatically in immunocompromised patients, during the past several decades. However, a few of yeast and yeast-like species may colonize in skin and mucous membranes of healthy individuals.

Objectives: The current study aimed at accurately identifying yeast and yeast-like species from clinical samples by molecular methods.

Methods: A total of 1200 clinical samples were collected from patients with suspected fungal infection and 110 (9.16 %) yeast and yeast-like strains isolated and identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), PCR amplification of *hwp1* gene, and sequencing.

Results: In total, *Candida albicans* (n = 46) was the most frequently isolated species followed by *C. parapsilosis* complex (n = 17), *C. tropicalis* (n = 13), *C. guilliermondii* (n = 12), *C. glabrata* (n = 4), *C. krusei* and *C. famata* each (n = 3), *C. kefyr*, *C. haemulonii* and *Cutaneotrichosporon jirovecii* each (n = 2), and *C. stellatoidea*, *C. intermedia*, *C. sorbosivorans*, *C. africana*, *Pichia kudriavzevii*, and *Trichosporon asahii* each (n = 1). Interestingly, *C. haemulonii*, a multidrug resistant fungus was isolated from cutaneous and sputum samples for the first time in Iran.

Conclusions: Nowadays, with growing population at risk for fungal infections and the emergence of some less virulent or non-pathogenic and uncommon yeasts not readily distinguishable with phenotypic assays, the accurate identification using molecular methods are warranted.

Keywords: Uncommon Yeasts, PCR-RFLP, *Candida haemulonii*, *C. sorbosivorans*, *hwp1* Gene

1. Background

The prevalence of fungal infections caused by yeasts and yeast-like species, particularly the genus of *Candida*, significantly increased due to extensive application of immunosuppressive drugs, predisposing factors, and particular conditions. Some rare and uncommon yeast species emerged as a cause of outbreak with high mortality or mixed infections non-responsive to routine antifungals (1, 2). Clinical manifestations of yeasts and yeast-like infections ranging from mild superficial to systemic infections are frequently and usually benign in immunocompetent individuals (3). *Candida* species are the major cause of yeast infections and *Candida albicans* is still the most frequently isolated yeast pathogen (4).

Non-*albicans Candida* species, e.g., *C. tropicalis*, *C. para-*

psilosis, *C. krusei*, *C. glabrata* and some uncommon yeast species such as *Trichosporon* and more specifically *C. auris* complex (*C. auris*, *C. haemulonii*, *C. pseudohaemulonii*, *C. duobushaemulonii*, and *C. haemulonii* var. *vulnera*) with a reduced susceptibility to antifungal agents become a serious clinical challenge and thus the isolates need to be properly identified (1, 2, 5-7). *Candida auris* and its closely related *C. haemulonii* emerged as a causative agent of invasive candidiasis that were extensively drug-resistant in hospital outbreaks (8, 9). *Candida auris* is often misidentified with *C. haemulonii* by phenotypic methods. Due to the high degree of similarity between *C. auris* complex, identification is problematic. Morphological and physiological approaches to identify the species are time-consuming and frequently unspecific (6, 10).

2. Objectives

The current study was aimed to determining the prevalence of common and uncommon yeasts particularly *Candida* species isolated from patients with candidiasis referred to medical mycology labs, and identifying the species using molecular methods that cannot be readily differentiated with phenotypic assays.

3. Methods

3.1. Ethics Statement

The current study was approved by the Ethics Committee of Fasa University of Medical Sciences (ethical code: 93210/D,97,247016), Fasa, Iran, and written informed consent was obtained from the patients.

3.2. Yeast Isolates

Totally, 1200 clinical samples were collected from patients suspected of fungal infection (age range 1- 84 years) referring to the medical mycology laboratory, from 2012 to 2014 as part of routine medical inquiry. In total, 110 yeast and yeast-like species were isolated from the nail (n = 46), cutaneous (n = 37), bronchoalveolar lavage (BAL) (n = 9), sputum (n = 7), mucosal specimens (n = 3), vagina (n = 2), ear discharge (n = 1), and urine (n = 1) samples. Isolates were initially examined for clinical specimens by direct microscopic examination (10% KOH), afterward cultured on malt-extract agar (Difco, USA) and incubated at 35°C for two days. The grown yeasts within 48 hours were initially identified by germ-tube test, and CHROMagar *Candida* medium (CHROMagar Company, Paris, France) as previously described (11).

3.3. Molecular Identification

By glass bead disruption method, genomic DNA was extracted as previously described and stored at -20°C prior to use (12). The PCR-RFLP method was conducted as previously explained (1). In a nutshell, PCR amplification of ITS1-5.8S-ITS2 rDNA regions was carried out in a final volume of 25 µL using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Metabion, Germany) (13). Each reaction consists of 1 µL template DNA, 1 µL of each primer at 10 pmol, 12.5 µL MasterMix (Ampliqon, Denmark) containing 5 mM dNTP, 0.5 U Taq DNA polymerase, and 10X PCR buffer and 9.5 µL double-distilled water. PCR conditions were adjusted with cycles of five minutes at 94°C for primary denaturation, followed by 35 cycles at 94°C (30 seconds), 55°C (45 seconds) and 72°C (60 seconds), and final extension at 72°C for seven minutes. Subsequently, PCR products were digested in a final reaction volume of 30 µL containing 17 µL water, 2 µL of

10X FastDigest buffer, 1 U of FastDigest restriction enzyme *MspI* (Fermentas, Thermo Fisher Scientific Inc., USA) and 10 µL PCR product at 37°C for 10 minutes. After the digestion with *MspI* enzyme, the PCR products were evaluated based on the molecular sizes by gel electrophoresis for *Candida* species according to Mohammadi et al. (1). *Candida albicans* CBS 562, *C. krusei* ATCC 6258, and *C. parapsilosis* ATCC 22019 were used as standard strains in the study.

In addition, the partial amplification of the hyphal wall protein 1 (*hwp1*) gene was used to differentiate *C. albicans* species complex (*C. africana*, *C. albicans*, *C. dubliniensis*, and *C. stellatoidea*) as described by Romeo and Criseo (14). Briefly, PCR amplification of *hwp1* gene was performed using the forward (5'-GCT ACC ACT TCA GAA TCA TCA TC-3') and reverse primers (5'-GCA CCT TCA GTC GTA GAG ACG-3') (Metabion, Germany) by producing three different DNA fragments as follows: 941 bp for *C. albicans*, ~740 bp for *C. africana*, 569 bp for *C. dubliniensis*, and ~800 bp for *C. stellatoidea* type I (14, 15). Amplified and digested products were stained with KBC power load (Kawsar Biotech Co., Iran) and run on 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 visualized by gel document device (12). *Candida albicans* CBS 562 and *C. africana* CBS 8781 were applied as standard strains in the study. For undifferentiated species by PCR-RFLP, sequencing was performed on an ABI 3730xl automatic sequencer (Applied Biosystems, Foster City, CA) using the primers ITS1 and ITS4, and for confirmation *C. africana hwp1* gene was amplified. Sequence data were obtained and compared with GenBank database using the basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov>). All obtained sequences were deposited in GenBank under the accession numbers KY091885, KY112734-KY112744, and KY238037-KY238038.

4. Results

Table 1 shows distribution of yeast species based on the site of infection in patients referring to the Mycology Lab of Baghban Clinic, Sari, Iran. In the current study, of 1200 clinical samples, 110 (9.16 %) were positive for yeast and yeast-like infections, of which 70 (63.6%) samples were obtained from females and 40 (36.4%) from males. Most of the samples were attributed to nail (41.8%), followed by skin (32.7%) and BAL (8.2%). Thirty-seven (80.4%) yeast species isolated from nail samples belonged to females. As shown in Table 2, depending on the species, the size of ITS rDNA region before and after endonuclease digestion with *MspI*, amplification of the ITS region in all yeast isolates yielded the band of approximately 389 to 881 bp.

Figure 1 showed the patterns of ITS PCR-RFLP for yeast isolates after digestion with *MspI*. The accurate identifica-

Table 1. Distribution of Yeast Species Based on the Site of Infection in the Current Study Subjects

Specimen	Nail	Skin	BAL	Sputum	Mouth	Urine	Vagina	Mucosa	Ear Discharge	Total
<i>Candida albicans</i>	20	8	7	5	4	1	1			46
<i>C. parapsilosis</i>	10	6							1	17
<i>C. tropicalis</i>	7	5	1							13
<i>C. guilliermondii</i>	7	5								12
<i>C. glabrata</i>	1	1		1	1					4
<i>C. krusei</i>			1					2		3
<i>C. famata</i>		3								3
<i>C. kefyr</i>	1	1								2
<i>C. haemulonii</i>		1		1						2
<i>Cutaneotrichosporon jirovecii</i>		2								2
<i>C. intermedia</i>		1								1
<i>C. sorbosivorans</i>		1								1
<i>C. africana</i>		1								1
<i>C. stellatoidea</i>							1			1
<i>Pichia kudriavzevii</i>								1		1
<i>Trichosporon asahii</i>		1								1
Total	46	36	9	7	5	1	2	3	1	110

Table 2. Size of ITS Region for Pathogenic Yeast Isolates Before and After Endonuclease Digestion With *MspI*

Yeast Species	Size of ITS	Size of <i>MspI</i> -RFLP Fragment
<i>Candida albicans</i>	537	239, 298
<i>C. parapsilosis</i>	530	530
<i>C. tropicalis</i>	526	186, 340
<i>C. glabrata</i>	881	320, 561
<i>C. krusei</i>	510	250, 260
<i>C. guilliermondii</i>	607	82, 155, 370
<i>C. famata</i>	639	639
<i>C. kefyr</i>	720	720
<i>C. intermedia</i>	389	122, 267
<i>C. haemulonii</i>	~ 400	~ 400
<i>C. sorbosivorans</i>	~ 440	~ 100, ~ 340

tion of the strains remained undifferentiated based on the results of sequencing method.

Figure 2 demonstrates the patterns of PCR amplification of *hwp1* gene for *C. albicans* complex isolates. Sequencing results from *C. africana* strain showed 99% similarity with those of GenBank database using BLAST. There were two different patterns of PCR amplification of *hwp1* gene for *C. albicans* (Figure 2; lanes 3 and 4), which Lane 3 is an atypical isolate of *C. albicans*, since they exhibited heterogeneous genetic characteristics (14).

Based on the above mentioned molecular methods, *C. albicans* was the most frequently isolated species (n = 46;

41.8%) followed by *C. parapsilosis* complex (n = 17; 15.4%), *C. tropicalis* (n = 13; 11.8%), *C. guilliermondii* (n = 12; 10.9%), *C. glabrata* (n = 4; 3.6%), *C. krusei* and *C. famata* (n = 3 each; 2.7%), *C. kefyr*, *C. haemulonii* and *Cutaneotrichosporon jirovecii* (n = 2 each; 1.8%) and *C. stellatoidea*, *C. intermedia*, *C. sorbosivorans*, *C. africana*, *Pichia kudriavzevii* (teleomorph of *C. krusei*), and *Trichosporon asahii* (n = 1 each; 0.9%). As shown in Table 1, several uncommon and less reported yeasts such as *C. africana* and *T. asahii* isolated from inguinal region, *P. kudriavzevii* from stomach mucosa, and two *C. jirovecii* from cutaneous samples were identified; furthermore, two cases of *C. haemulonii* (KY112737 and KY112738) and *C. sorbosivorans* (KY112736) were reported for the first time in Iran. It is noteworthy that *C. haemulonii* (S49AF, accession number: KY112738) strain was reconfirmed by a novel multiplex end-point PCR as well (16).

5. Discussion

Among hundreds of yeast and yeast-like species, only a few are the normal flora of skin and mucous membranes. These yeasts may cause opportunistic infections in immunocompromised patients under particular conditions (17). In epidemiological studies, most of the yeast isolates belong to *Candida* species, especially *C. albicans* (1, 4, 5, 17, 18). Previous studies in different regions of Iran show that *Candida* species were isolated from various clinical samples rather than other yeasts (2, 5, 18-23). As already mentioned in most studies, among *Candida* species, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* were the

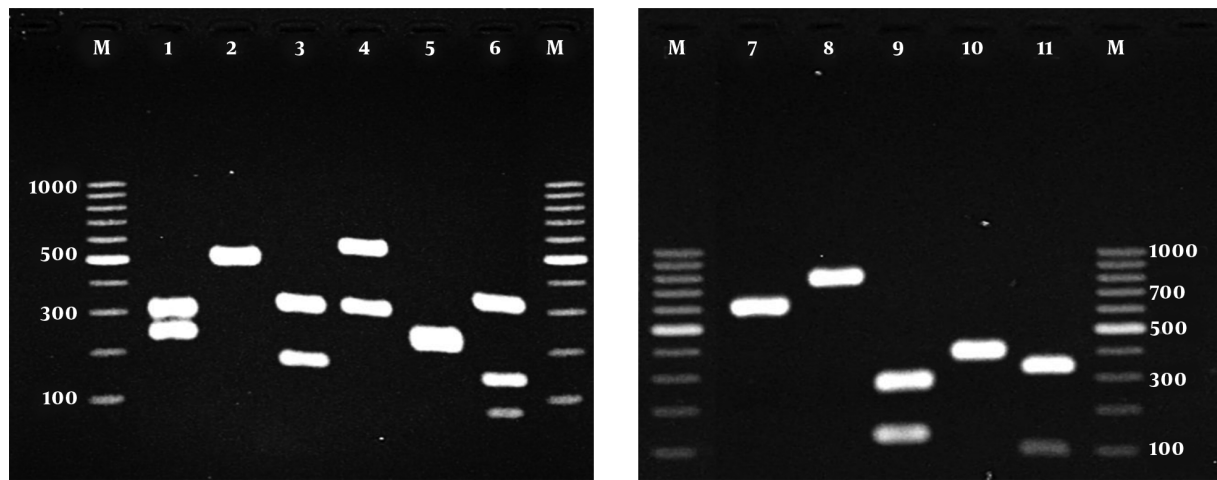


Figure 1. Agarose gel electrophoresis of ITS-PCR products of yeasts species after digestion with *MspI*; lanes 1 - 11: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. famata*, *C. kefyr*, *C. intermedia*, *C. haemulonii* and *C. sorbosivorans*, respectively; Lane M: a 100-bp DNA size marker

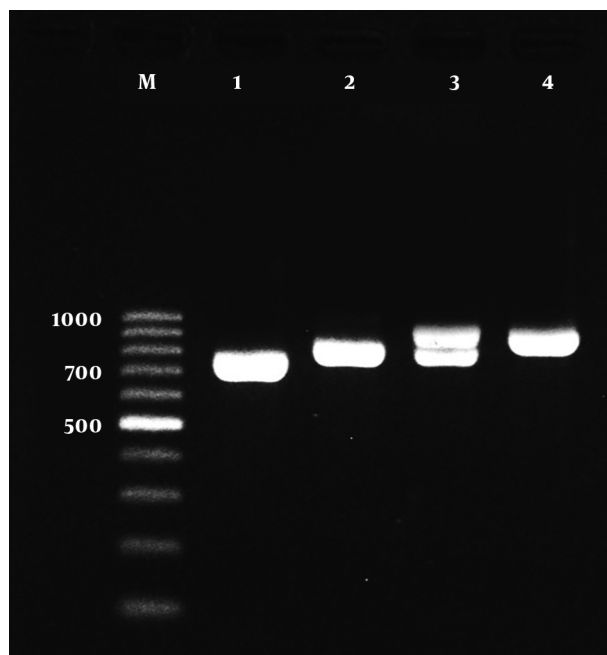


Figure 2. Species-specific amplification of the *hwp1* gene; Lane 1 for *C. africana*, Lane 2 for *C. stellatoidea*, and lanes 3 and 4 for *C. albicans*; Lane M, a molecular size marker.

most common causes of candidiasis in Iran. Several studies showed that *Candida* onychomycosis was the predominant yeast infection (2, 5, 19, 21), consistent with the current study results (41.8%). In the current study, the most prevalent species isolated from clinical samples was *C. albicans*, followed by *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii*. Moreover, some uncommon yeasts including

C. africana, *T. asahii*, and *C. jirovecii* isolated in the current study that were less reported from Iran so far (24-27).

In recent years, there are some reports of infections caused by uncommon yeasts and yeast-like fungi in the world. For instance, Kubica et al., reported the isolation of 30 *T. asahii* strains from clinical samples in Brazil (28). Messias Silvestre et al., isolated 112 *Trichosporon* spp. from perigenital skin with predominance of *T. cutaneum*, and 30 *Trichosporon* species strains from urine samples and catheters, mainly *T. asahii* (29). Chagas-Neto et al., recovered 22 isolates from blood cultures, mostly *T. asahii* (30). A study from Italy reported 17 cases of invasive infections caused by *Trichosporon* species (31). Almeida Junior et al., comprehensively reviewed 203 cases of invasive *Trichosporon* infection in the literature reported from 1994 to 2015 indicating that *T. asahii* was the predominant species (46.7%) caused trichosporonosis (7). Moreover, Karimi et al. (32), identified 23 uncommon yeasts out of 855 (5.7%) yeast strains isolated from Iran; yeast species not reported in Iran so far (e.g., *C. magnolia*) are closely related with *C. sorbosivorans* in terms of phylogenetic relationships (33). In the current study, *C. sorbosivorans* (KY112736) was isolated from inguinal region of a 55-year-old female. To the best of our knowledge, it was the first report of *C. sorbosivorans* as a causative agent of candidiasis. *Candida sorbosivorans* isolated from contaminated industrial material for the first time by James et al. (33).

As well as, for the first time *C. haemulonii* (KY112737-38) was reported from Iran, from a 36-year-old male with cutaneous candidiasis and the sputum of a 75-year-old male. This species reconfirmed thereafter using a novel multiplex (16). *Candida haemulonii* and its close relative *C. au-*

ris caused remarkably high mortality, especially in patients with candidemia (8, 9). *Candida haemulonii* as a member of *C. auris* complex emerged as an opportunistic fungal pathogen associated with candidiasis. Some species related to *C. auris* complex were responsible for candidemia and showed antifungal resistance profiles (10). Other members of *C. auris* complex include *C. pseudohaemulonii*, *C. duobushaemulonii*, *C. haemulonii* var. *vulnera*, and *C. auris* (10, 34-37). In most studies, antifungal susceptibility tests of *C. auris* complex show high resistance to azole derivatives, especially fluconazole; but echinocandins are highly active against *C. auris* complex (10, 36, 38-40). However, strains resistant to this new class of antifungal agents were also reported (41, 42).

5.1. Conclusions

With the growing population at risk for fungal infections and the emergence of some less virulent or non-pathogenic and uncommon yeasts not readily distinguishable with phenotypic assays, the accurate identification of such microorganisms using molecular methods are warranted.

Footnotes

Authors' Contribution: Seyed Mohammad Hosein Afsarian: Study concept and design, data collection, analysis, and interpretation, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, administrative, technical, and material support, and study supervision; Tahereh Shokohi: Study concept and design, and critical revision of the manuscript for important intellectual content; Nasim Moradi and Laleh Baderam: Analysis and interpretation of data; Hamid Badali and Mohammad Reza Ataollahi: Critical revision of the manuscript for important intellectual content.

Conflict of Interests: No potential conflict of interests.

Funding/Support: The authors gratefully appreciate the Research Deputy of School of Medicine, Fasa University of Medical Sciences, Fasa, Iran for the financial support of the current study.

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