Original Article

Molecular identification of uncommon clinical yeast species in Iran

Karimi L[1], Mirhendi H[2]*, Khodadadi H[3], Mohammadi R[4]

[1] Dr. Beheshti Hospital, Social Security Organization, Shiraz, Iran
[2] Department of Medical Parasitology and Mycology, School of Public Health, National Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran
[3] Department of Medical Parasitology and Mycology, School of Medicine; Shiraz University of Medical Sciences, Shiraz, Iran
[4] Department of Medical Parasitology and Mycology, School of Medicine; Isfahan University of Medical Sciences

***Corresponding author: Hossein Mirhendi**, Department of Medical Parasitology and Mycology, School of Public Health, National Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran. Tel: 021 8895 1392; Email: mirhendi@tums.ac.ir

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Abstract

Background and Purpose: By using advanced detection/identification methods, the list of emerging uncommon opportunistic yeast infections is rapidly expanding worldwide. Our aim in the present study was sequence-based species delineation of previously unidentified yeasts obtained from a clinically yeast collection.

Materials and Methods: A total of twenty three out of the 855 (5.7%) yeast isolates which formerly remained unidentified by PCR-RFLP method, were subjected to sequence analysis of the entire internal transcribed spacers (ITS) regions of rDNA. The precise species recognition was performed by the comparison of the sequences with the reliable GenBank database.

Results: Sequencing analysis of the ITS region of the strains revealed several uncommon yeasts that were not reported previously in Iran. The species include *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Sporidiobolus salmonicolor*, *Pichia fabianii*, *Pichia fermentans*, *Candida famata*, *Candida inconspicua*, *Candida maqnoliae*, *Candida guilliermondii*, *Candida kefyr*, *Candida rugosa*, *Candida lusitaniae*, *Candida orthopsilosis*, and *Candida viswanathii*.

Conclusion: We identified several rare clinical isolates selected from a big collection at the species level by ITS-sequencing. As the list of yeast species as opportunistic human fungal infections is increasing dramatically, and many isolates remain unidentified using conventional methods, more sensitive and specific advanced approaches help us to clarify the aspects of microbial epidemiology of the yeast infections.

Keywords: Iran, Molecular typing, Yeasts

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Introduction

Among hundreds of yeast species, only a few may colonize mucocutaneous membranes of healthy individuals and in specific circumstances they can cause opportunistic infections in immunocompromised patients [1]. Although *Candida albicans* and *Cryptococcus neoformans* are the foremost causes of yeast infections worldwide, recently some non- *albicans Candida* and non-*Cryptococcus* species have emerged [2-3]. Although at present, the frequency of these species is uncommon or rare; it is possible that after antifungal controlling of more susceptible species, they become more frequent in the future. These emerging yeast species can cause severe opportunistic infections in predisposed hosts [3-4]. Due to the high similarity of the related species and numerous limitations of conventional yeast identification methods, these sorts of opportunistic yeasts often are misidentified or reported only as unknown yeast species. On the other hand, some of these species show inherent or acquired resistance to available antifungal agents [5-6]. Therefore, the identification and determination of their susceptibility profiles to antifungal drugs is necessary for the successful management of the patients and the control of infection in the nosocomial as well as the community-acquired settings. During recent decades, advanced molecular techniques including molecular identification approaches mostly based on polymerase chain reaction (PCR), i.e., PCR-RFLP, real-time PCR or other techniques have been introduced to overcome the limitations of phenotypic methods of yeast recognition [7-8].

These techniques are obviously more sensitive and faster than conventional techniques, however, they are not efficiently able to differentiate all yeast species [9].

DNA-sequencing of suitable target as the most accurate molecular method has expanded the list of emerging yeasts [10-12]. The analysis of sequences of the ribosomal RNA genes (rDNA) especially the internal transcribed spacer (ITS) region has allowed us a better discrimination of rare yeast species. The higher sensitivity and specificity of these techniques have revealed those uncommon yeasts which had already been ignored [11, 13]. For all that, the accurate identification of yeasts based on species level often needs a combination of conventional and molecular methods [14].

We have recently collected and identified 855 yeast strains, isolated from various clinical forms of infections, during which the precise identity of some isolate remained undetermined [15]. The aim of the current study was to identify these uncommon yeasts at the species level by PCR-sequencing as the most precise method. Interestingly, we found some rare yeast that to the best of our knowledge had not previously been reported in Iran.

Material and Methods

The strains used in the study were a part of a large collection which has already isolated from various clinical specimens including skin, nails, bronchoalveolar fluid, sputum, vaginal discharges, blood, urine and stools; originated from Tehran, Esfahan, Alborz and Mazandaran provinces [15]. To identify the species, the isolates had already subjected to previously described PCR-RFLP method [8-9, 15]. A total of 23 yeasts which remained unidentified by PCR-RFLP were included in the present study.

The yeasts were sub cultured on Sabouraud dextrose agar (Merck KGaA, Darmstadt, Germany) and Chromogenic medium (CHROMagar Candida, Paris, France) and incubated at 30°C for 48 h. Genomic DNA of each fresh colony was isolated using FTA Elute MicroCards (Whatman Inc., Clifton, NJ, USA) as described elsewhere [15]. As the DNA sequence of different parts of ribosomal DNA (rDNA), particularly the internal transcribed spacer (ITS1-5.8S-ITS2) region proved as the valuable molecular markers for sequence identification of yeast species, all isolates were conducted to ITS-PCR-sequencing [16]. Each PCR mixture contained 12.5 µl 2× Master Mix RED (Ampliqon, Denmark), 0.25 µM of each forward (ITS1: 5´-TCCGTAGGTGAACCTGCGG-3´) and reverse (ITS4: 5´- TCCTCCGCTTATTGATATGC- 3´) universal fungal primers [17], 2µl of extracted DNA and enough ultrapure water up to a final reaction volume of 25 µl. The thermal cycler was programmed for an initial denaturation at 95 for 5 min and 35 cycles of 94°C for 45s, 56°C for 45s and 72°C for 1 min and a final extension step at 72°C for 10 min. Five micro liter of the PCR products were electrophoresed onto 1.2% agarose gel in TBE buffer (Tris 90mM, Boric acid 90mM, EDTA 2mM), stained with 0.5 µg/ml of ethidium bromide and observed and photographed under UV light. PCR products were purified using a PCR purification kit (Bioneer, Korea) and directed to an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and sequenced using an automated DNA Sequencer (ABI Prism 3730Genetic Analyzer; Applied Biosystems). Subsequently, the final designation for species was based on the analysis of reliable sequences with BLASTn algorithm in comparison with relevant reliable sequences deposited in GenBank (http: // www. ncbi.nlm.nih.gov/BLAST).

The yeast species were also identified by a newly designed method namely fragments size polymorphism (PCR-FSP) of the ITS1 and ITS2 regions [18]. Briefly, the genomic DNA of isolates was PCR-amplified using two primer pairs (ITS1-ITS2 and ITS3-ITS4) [17] separately. Both PCR products were mixed and analyzed after electrophoresing on 2% agarose gel. The species of the tested yeasts were delineated by the electrophoretic patterns of the mixed PCR products of each sample, comparing the data obtained from the sequence analyses of mean size of ITS1 and ITS2 molecules found in GenBank library [18].

Results

Among the 855 yeast strains which were previously isolated from patients with various forms of fungal infections and identified by PCR-RFLP, a total of 23 yeasts were classified as uncommon yeast species, and none of them showed a specific color of dominant yeasts on Chromogenic plates. These isolates were subjected to ITS rDNA sequencing and subsequently analyzed for homology with other reliable sequences deposited in GenBank. Table 1 summarized the results of this analysis.

Among the isolates, we found six new yeast species which have not reported in Iran so far, including two strains of *Hanseniaspora uvarum*, one strain of *Pichia fabianii*, one *Sporidiobolus salmonicolor*, one *Pichia fermentans* and one *Candida magnolia*. Three out of 23 (13%) isolates were correctly identified by PCR-RFLP method (two *Candida-opsilosis* complex and one *C. tropicalis*); the method could not discriminate 17 remaining isolates and reported them as unidentifiable. According to the results of sequencing, three isolates were reported falsely by PCR-RFLP (Table1).

Fourteen out of 23 isolates were correctly identified by PCR-FSP method. These strains were: two *Candida kefyr*, two *C. rugosa*, four *C. lusitaniae*, one *C. -opsilosis* complex, three *Saccharomyces cerevisiae*, one *C. inconspicua* and one *C. guilliermondii*. By PCR-FSP method 7 isolates were misjudged and two were reported as unidentifiable (Table1).

Table 1. Identified clinical yeast isolates through different yeast identification methods

Sample ID	Source	Age	Sex	Species identified by Sequencing (GenBank accession number)*	Species identified by FSP	Species identified by RFLP
1	BAL	55	M	*Candida kefyr*	*Candida kefyr*	Unidentified
2	Stool	60	M	*Candida kefyr*	*Candida kefyr*	Unidentified
3	Skin	32	F	*Candida rugosa*	*Candida rugosa*	Unidentified
4	Nail	44	M	*Candida rugosa*	*Candida rugosa*	Unidentified
5	Blood	63	F	*Candida lusitaniae* (KP204933)	*Candida lusitaniae*	*Candida parapsilosis*
6	Nail	33	F	*Candida lusitaniae* (KP204936)	*Candida lusitaniae*	Unidentified
7	Nail	88	F	*Candida lusitaniae*	*Candida lusitaniae*	*Candida krusei*
8	Nail	63	F	*Candida lusitaniae*	*Candida lusitaniae*	Unidentified
9	Nail	57	F	*Candida tropicalis* (KP204932)	*Candida viswanathii*	*Candida tropicalis*
10	Nail	60	F	*Candida orthopsilosis* (KP204937)	*Candida viswanathii*	Unidentified
11	Nail	3	M	*Candida parapsilosis*	*Candida parapsilosis*	*Candida parapsilosis*
12	Nail	-	F	*Hanseniaspora uvarum* (KP204941)	*Candida nivariensis*	Unidentified
13	Nail	50	F	*Hanseniaspora uvarum* (KP204943)	*Candida nivariensis*	Unidentified
14	Vaginal discharge	37	F	*Candida parapsilosis* (KP204942)	*Candida nivariensis*	*Candida parapsilosis*
15	Nail	8	M	*Saccharomyces cerevisiae* (KP204934)	*Saccharomyces cerevisiae*	Unidentified
16	Nail	36	F	*Saccharomyces cerevisiae* (KP204935)	*Saccharomyces cerevisiae*	Unidentified
17	Nail	73	M	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	Unidentified
18	Sputum	69	M	*Pichia fabianii* (KP204938)	*Candida famata*	*Candida tropicalis*
19	Sputum	26	F	*Sporidiobolus salmonicolor* (KP204944)	Unidentified	Unidentified
20	BAL	57	M	*Pichia fermentans* (KP204939)	*Candida inconspicua*	Unidentified
21	Skin	16	F	*Candida maqnoliae* (KP204940)	Unidentified	Unidentified
22	Nail	32	F	*Candida inconspicua*	*Candida inconspicua*	Unidentified
23	Nail	54	M	*Candida guilliermondii*	*Candida guilliermondii*	Unidentified

**Some sequences did not submit in GenBank*

Discussion

With growing population at risk for fungal infection and emerging some lesser virulent or non-pathogenic fungi, the increased rate of recovering uncommon yeasts from clinical samples may be partly have attributed to the use of more sensitive and specific diagnostic methods during the recent decades [19-20].

Diagnosis of yeast infections routinely depends on conventional microbiological methods especially morphological and physiologic methods, however, these approaches do not have enough sensitivity and specificity to correctly discriminate all yeast species [21]. Some nucleic acid-based methods like PCR-RFLP have improved detection and identification of clinical yeast isolates [7]. Nevertheless, the discriminatory power of this method severely depends on the digestion profiles and number of used restriction enzymes [8-9]. Therefore the application of this method was not able to differentiate all yeast species and consequently may lead to misjudging or misidentifying some newly emerging or uncommon yeast species. Recently, some new technologies like matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has adapted to identify yeast species in clinical settings [14, 22-23]. Although the use of this technique is a big advancement in the delineation of yeasts, this method is dependent on the already established data library and has its own limitations and at present cannot differentiate all yeast species especially those newly emerging yeasts. The method has shown a possibility of misidentification of some uncommon yeasts and poor ability to identify some species like *C. lusitaniae*, an amphotericin B resistant species that may be clinically important [24].

Some *Candida* species including *C. albicans, C. glabrata, C. tropicalis, C. krusei* and *C. parapsilosis* are dominant yeasts which routinely are isolated from clinical samples, while other species are rarely recovered from clinical specimens [25-26]. In this study, we aimed to screen uncommon yeasts that remained unidentified or misidentified with conventional or simple PCR-based methods (PCR-RFLP and PCR-FSP). We screened a total of 23 strains from the yeast isolates which had already been collected in our laboratory. Definitive identification was carried out by sequencing of the entire internal transcribed spacer region compared with verified GenBank sequences [13].

Although PCR-RFLP method used in our study showed a strong power for discrimination of the common and dominant yeasts and 832 out of 855 isolates were identified undoubtedly by this method, but this method showed a poor ability to straight-forward identifying of some uncommon yeasts. The evidence of inability of this method for discriminating rare yeasts is the lower concordance between PCR-RFLP and sequencing results (13%) among 23 isolates identified by both methods. In comparison with PCR-RFLP, PCR-FSP showed a better power for discriminating rare yeasts and had a better concordance with sequencing results (60.9%). Discordance of PCR-RFLP and FSP methods was dominant (83.3%) among 6 isolates identified by both methods. Altogether the conventional and two PCR-based methods used in this study showed the inability to identify uncommon yeasts correctly as indicated previously by other researchers [5, 12, 21].

In the current study for the first time in Iran we reported rare and uncommon yeast that some of them had not been isolated or reported from clinical samples previously. These include some rare species like *Hanseniaspora uvarum, Saccharomyces cerevisiae, Sporidiobolus salmonicolor, Pichia fabianii, Pichia fermentans* and some uncommon *Candida* species like: *Candida famata, Candida inconspicua, Candida maqnoliae, Candida guilliermondii, Candida kefyr, Candida rugosa, Candida lusitaniae* and *Candida orthopsilosis.*

As mentioned previously, we used a large yeast collection without designing a systematically program to collect our samples and they were randomly included in our study [15]. Thus, the rate of newly identified uncommon yeast infections in this study is not the exact representative of the frequency of uncommon yeast infections in Iran. However, isolation and identification of these yeasts from clinical specimens indicate the existence of

newly emerging yeast infection in at risk Iranian patients, even though these infections often neglect as a result of their misidentification.

Almost all these rare or uncommon yeast spices had been mentioned in some studies from the other parts of the world. They have been identified by different methods of yeast identification or on various groups of patients. On the other hand, there is not an exact estimation about the worldwide rate of these opportunistic infections; they are randomly identified and reported as new case reports [1, 3, 12, 19, 21].

As some of these yeasts have shown inherent and acquired resistance to some antifungal drugs, antifungal susceptibility testing is recommended to diagnostic laboratories. In conclusion, in this study we identified several rare clinical isolates selected from a big collection, at the species level, by ITS-sequencing. As the list of yeast species as opportunistic human fungal infections is increasing dramatically, and many isolates remain unidentified using conventional methods, more sensitive and specific advanced approaches are recommended to clarify the aspects of microbial epidemiology of the yeast infections.

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Authors' contributions

H.M. designed and managed the research, edited the final manuscript. L.K. and R.M. performed the tests. H.K. analyzed data and wrote the manuscript.

Conflicts of interest

Authors declare that there is no conflict of interest.

Financial disclosure

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

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