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Rapid detection of *Candida* species in bronchoalveolar lavage fluid from patients with pulmonary symptoms

Hossein Zarrinfar[a,b], *Saeed Kaboli*[c], *Somayeh Dolatabadi*[d,e,f], *Rasoul Mohammadi*[g,*]

[a] *Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[b] *Department of Medical Parasitology and Mycology, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[c] *Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan*
[d] *Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar, Iran*
[e] *CBS-KNAW, Utrecht, The Netherlands*
[f] *Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands*
[g] *Department of Medical Parasitology and Mycology, School of Medicine and Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*

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ABSTRACT

Candida species, especially *C. albicans*, are commensals on human mucosal surfaces, but are increasingly becoming one of the important invasive pathogens as seen by a rise in its prevalence in immunocompromised patients and in antibiotic consumption. Thus, an accurate identification of *Candida* species in patients with pulmonary symptoms can provide important information for effective treatment. A total of 75 clinical isolates of *Candida* species were obtained from the bronchoalveolar lavage fluid of both immunocompromised and immunocompetent patients with pulmonary symptoms. *Candida* cultures were identified based on nuclear ribosomal Internal Transcribed Spacer (ITS1-ITS2 rDNA) sequence analysis by polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP). Molecular identification indicated that the isolates belonged predominantly to *C. albicans* (52%), followed by *C. tropicalis* (24%), *C. glabrata* (14.7%), *C. krusei* (5.3%), *C. parapsilosis* (1.3%), *C. kefyr* (1.3%) and *C. guilliermondii* (1.3%). Given the increasing complexity of disease profiles and their management regimens in diverse patients, rapid and accurate identification of *Candida* species can lead to timely and appropriate antifungal therapy.

* *Corresponding author at*: Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: dr.rasoul_mohammadi@yahoo.com (R. Mohammadi).
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Introduction

Candida spp. are the causative agents in 80% of nosocomial fungal infections.[1] They are frequently isolated from respiratory secretions in mechanically ventilated patients due to either seeding of the lungs during hematogenous dissemination, or aspiration of previously colonized oropharyngeal or gastric contents.[2] Although the isolation of *Candida* spp. from the bronchoalveolar lavage (BAL) fluid of immunocompetent individuals is not an indication for treatment,[3] nevertheless, 24% of all intensive care physicians prescribe antifungal therapy in immunocompetent, mechanically ventilated patients testing positive for *Candida* spp.[4] Although the accuracy of tracheal surveillance cultures is controversial, the absence of *Candida* spp. in these cultures has a high negative predictive value for disseminated candidiasis in patients with leukemia, lymphoma, or those who have undergone bone marrow transplantation.[2] Rapid identification of *Candida* infections can, therefore, help in prompt and appropriate antifungal therapy. However, the diagnosis of pulmonary candidiasis is still controversial,[5] and the detection of primary fungal lung infection requires a lung biopsy.[6] However, in routine clinical practice, lung biopsies cannot be used for the management of patients with suspected *Candida* infection.[7]

Although *C. albicans* is the most frequently isolated pathogen,[8] an increase in the incidence of infections due to other isolates, including *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, which are the cause of opportunistic infection oropharyngeal candidiasis (OPC), has been reported.[9] In recent decades, *C. glabrata*, which is resistant to fluconazole, has emerged as the second most common causative organism (10–30% of all yeast isolates) of mucosal and invasive fungal infections,[10] trailing only *C. albicans* (50–60%). A large multicenter study showed an increase in the occurrence of *C. glabrata* infections from 14% in 1993 to 24% in 1998.[10] The reasons for this increase have been investigated, and include shifts in the distribution of infections by certain *Candida* spp. and along with both endogenous and exogenous reservoirs.[11] Therefore, an accurate identification of the causative *Candida* spp. in patients with pulmonary symptoms, particularly in immunocompromised individuals, will enable proper diagnosis and effective treatment, apart from broadening the physicians' knowledge of the epidemiology of *Candida* spp.[5,12,13] Thus, this study aims to accurately identify and quantify the prevalence of *Candida* spp. present in the BAL fluid of both immunocompromised and immunocompetent patients with pulmonary disorders such as pulmonary candidiasis or colonization.

Materials and methods

Specimens and preparation

During a 16-month period, 75 BAL fluid specimens were obtained from both immunocompromised and immunocompetent patients with pulmonary disorders who were referred to the Shariati Hospital, Tehran, Iran for bronchoscopy. BAL specimens were submitted to the Medical Mycology laboratory at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. The BAL fluid specimens, requested and obtained by a specialist physician, were collected in sterile vessels without any conservative media and transferred to the laboratory. The starting volume of BAL fluid ranged between 4 and 7 mL. Specimens were centrifuged at 3000 rpm for 20 min, pellet vortexed and resuspended in a small volume of supernatant (total 0.5–1 mL).[14]

A 150 μL aliquot of the sediment was mixed with a drop of 20% potassium hydroxide (KOH) on a microscopic slide, covered with a large sterile coverslip (24 mm × 50 mm) and viewed under 100× and 400× magnifications. The aliquots of the specimens (75 μL) were plated on both 4% Sabouraud glucose agar (SGA) (Difco, USA) and Brain Heart Infusion agar (BHI) (Difco, USA).[15] Plates were incubated for 2–4 days at 30 °C and monitored for growth of yeast colonies. Colony isolates were suspended in sterile distilled water and kept at −4 °C until DNA extraction.

DNA extraction and molecular studies

To extract genomic DNA from the yeast colonies, FTA® Elute MicroCards (Whatman Inc., Clifton, NJ, USA) were used[16] according to manufacturer's instructions but with minor modifications as described by Mohammadi et al.[17] The extracted DNA samples were stored at −20 °C until use. Quality control was ensured by adding 18 reference strains of medically important yeasts to the dataset, supplied by the Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan. The reference strains used were *C. tropicalis* (ATCC 750), *C. albicans* (TIMM 1768), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 22019), *C. nivariensis* (CBS 10161), *C. bracarensis* (CBS 10154), *C. krusei* (ATCC 6258), *C. orthopsilosis*, *C. metapsilosis*, *C. guilliermondii* (TIMM 3400), *C. lusitaniae* (TIMM 3479), *C. famata* (JCM 1439), *C. kefyr* (TIMM 0300), *Cryptococcus neoformans* (ATCC 90113), *C. norvegensis* (JCM 2309), *C. inconspicua* (JCM 9555), *C. lusitaniae* (TIMM 3479), *C. intermedia* (JCM 1607), *C. rugosa* (JCM 1619), *C. viswanathii* (JCM 9567), *Saccharomyces cerevisiae* (ATCC 9763).

In addition, DNASIS software (Hitachi Software Engineering Co., Ltd, Tokyo, Japan) was used to analyze GenBank sequences of different yeasts to determine the size of the entire ITS1-5.8SrDNAITS2 region before and after *in silico* digestion with the *Msp* I restriction enzyme,[17] and molecular identification was performed based on PCR-RFLP profiles as described previously.[17–19] Briefly, the region was amplified using a PCR mixture containing 5 μL of the 10× reaction buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 2.5 U of DNA *Taq* polymerase, 30 pmol of each ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers and 3 μL of extracted DNA, in a final volume of 50 μL. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. An aliquot of 10 μL of each PCR product was digested with the restriction enzyme *Msp* I (Fermentas, Vilnius, Lithuania), and 12 μL of each RFLP product was fractionated by electrophoresis.

To confirm the validity of the molecular results for identification of common *Candida* spp., some isolates including *C. albicans, C. glabrata, C. krusei* and *C. tropicalis* were

subcultured on CHROMagar *Candida* (CHROMagar Microbiology, Paris, France) and incubated at 35 °C for 2–3 days.

Results

Seventy five isolates were obtained from BAL specimens of 32 immunocompromised and 43 immunocompetent patients. The immunocompromised patient profile included solid-organ cancers ($n = 8$), intensive care units (ICU) patients ($n = 8$), autoimmune conditions requiring corticosteroids ($n = 7$), solid organ transplant recipients ($n = 6$), bone marrow transplant recipients ($n = 3$).

Among these patients, 61% were male ($n = 46$) with an average age of 58.3 ± 17.6 years, and 39% were female ($n = 29$) with an average age of 50.5 ± 19.8 years. The immunocompetent patients had significant history of respiratory disorders such as cough, dyspnea, chest pain, hemoptysis, bronchial asthma, and the presence of a cavity within an area of consolidation on computerized tomography (CT) imaging. Table 1 summarizes the demographic characteristics of the patients.

Pseudohyphae were seen in 38 specimens and yeast forms were seen in 37 specimens upon direct microscopic examination.

The primer pairs (ITS1 and ITS2) were able to amplify the ITS region and provided a single PCR product. Isolates were identified using a combination of ITS-RFLP with *Msp*I and the ITS-amplicon size as previously established.[17]

Seven *Candida* species, including *C. albicans* (52%, $n = 39$), *C. tropicalis* (24%, $n = 18$), *C. glabrata* (14.7%, $n = 11$), *C. krusei* (5.3%, $n = 4$), *C. parapsilosis* (1.3%, $n = 1$), *C. kefyr* (1.3%, $n = 1$) and *C. guilliermondii* (1.3%, $n = 1$) were identified (Table 1).

Importantly, the results of species identification using CHROMagar *Candida* for selected *Candida* isolates concurred with those of the PCR-RFLP.

Table 1 – Demographics of enrolled patients.

Characteristic	Immunosuppressive condition	No. of BAL specimens	Male/female ratio	Mean age (range)	Age range	Isolated *Candida*					
						C. albicans	*C. tropicalis*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. kefyr*	*C. guilliermondii*
Immunocompetent hosts	–	43	28/15	61.7	16–86	22	12	5	3	0	1	0
Sub-categories of immunocompromised hosts	Solid-organ cancer patients	8	4/4	56.4	21–78	7	1	0	0	0	0	0
	Patients in ICU	8	4/4	39.7	20–73	4	1	2	0	1	0	0
	Patients with autoimmune diseases and corticosteroids consumer	7	4/3	46.4	34–75	3	3	0	0	0	0	1
	SOT recipients	6	4/2	48.5	45–55	3	0	3	0	0	0	0
	BMT recipients	3	2/1	36.7	20–48	0	1	1	1	0	0	0

BAL, bronchoalveolar lavage fluid; ICU, intensive care units; SOT, solid organ transplant; BMT, bone marrow transplant.

Discussion

Seventy-five *Candida*-positive isolates were obtained from immunocompromised and immunocompetent patients with pulmonary disorders and identified using PCR-RFLP. In recent decades, the prevalence of *Candida* infections has increased, particularly in immunocompromised patients. The incidence of *Candida* pneumonia varies in different studies, and ranges between 0.23% and 4.5%.[20,21] Although *Candida* spp. are frequently isolated from respiratory secretions of mechanically ventilated patients, its presence in respiratory specimens only represents colonization of the tracheobronchial tree, and the criteria for the diagnosis of pulmonary candidiasis are still controversial. In addition, the value of quantitative cultures of respiratory samples in diagnosing *Candida pneumonia* is unknown,[5] since this study had two limitations: low specimen number for evaluation of *Candida* prevalence and lack of histologic findings consistent with pulmonary candidiasis.

The potential clinical importance of species-level identification for *Candida* infections has been recognized as individual species difference in the expression of putative virulence factors and antifungal susceptibility.[22,23] In addition, rapid identification of *Candida* spp. can also lead to early and effective antifungal therapy, given the increasing rate at which

drug-resistant *Candida* spp. are being reported.[24,25] However, most yeast isolates obtained from patients with pulmonary disorders, especially from immunocompromised patients, can be identified to the species-level to reduce antifungal resistance among the *Candida* spp., and associated morbidity and mortality in these patients. Traditional diagnostic methods such as direct examination and culture are not sufficient for the identification of yeast isolates, and in this study, pseudohyphae and yeast forms were seen in different species upon direct microscopy examination, without any significant relationship between species identity and morphology. An exception to this observation is *C. glabrata* which has only the yeast form. Further, fungal cultures could not differentiate between the *Candida* species, while all isolates could be successfully identified using PCR-RFLP.

The results of the present study confirm that PCR-RFLP helps in the presumptive identification of *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. kefyr* and *C. guilliermondii*, and are similar to other reports.[17–19,26] Although we report a *C. albicans* prevalence of 52%, it is less than the reported value in other studies (70–80%).[27,28] This could be because, over the last 10–30 years, non-*C. albicans Candida* (NCAC) species have emerged as important opportunistic pathogens in humans, due to improved diagnostic methods, altered medical practices, differences in patient groups or geographic location.[28] Compared to other studies, we show higher incidence of *C. tropicalis* (24%) and *C. glabrata* (14.7%) and lower incidence of *C. parapsilosis* and *C. kefyr*,[17,29] while that of *C. guilliermondii* (1.3%) was similar.[17,30]

The intriguing observation in our study was the difference in the distribution of the *Candida* spp. among the immunocompromised and immunocompetent patients: immunocompetent patients showed higher incidence of *C. tropicalis*, *C. krusei*, *C. kefyr* and *C. albicans* and a lower incidence of *C. parapsilosis* and *C. guilliermondii*, compared to immunocompromised patients.

Thus, this study highlights that, *Candida* isolates can be successfully identified through PCR-RFLP and that such identification is important to show variation in the prevalence of *Candida* spp. among different patient groups, especially high risk patients with signs of clinical deterioration.

Conflict of interest

The authors declare no conflicts of interest.

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