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ORIGINAL ARTICLE

Identification and assessment of antifungal susceptibility of *Candida* species based on bronchoalveolar lavage in immunocompromised and critically ill patients

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ABSTRACT

Background and Objectives: The presence of fungi in the respiratory tract as mycobiome, particularly *Candida* species (spp.), remains a serious problem due to increasing numbers of immunocompromised patients. The confirmed reliable existence of these pathogens due to frequent colonization is essential. This investigation aimed to recognize *Candida* spp. among isolates from bronchoalveolar lavage of immunocompromised and critically ill patients and to evaluate their susceptibility to antimycotic drugs.

Materials and Methods: Bronchoalveolar lavage fluid was collected from 161 hospitalized patients presenting with suspected respiratory fungal infection /colonization. The specimens were examined by standard molecular and mycological assays. *Candida* spp. were recognized with sequence assessment of the D1-D2 section of the large subunit ribosomal DNA. The susceptibility of *Candida* isolates to common antimycotic drugs was distinguished by standard broth microdilution.

Results: Seventy-one clinical isolates of *Candida* spp. were recognized. *Candida albicans* was the most frequent, followed by *C. glabrata, C. krusei* (*Pichia kudriavzevii*), *C. dubliniensis, C. parapsilosis*, and *C. tropicalis*. We found 5.1% of *C. albicans* isolates and 8% of *C. glabrata* isolates to show resistance to fluconazole. The whole of the *Candida* spp. were sensitive to amphotericin B and caspofungin.

Conclusion: This study demonstrated that *C. albicans* and *C. glabrata* are the most common isolates of bronchoalveolar lavage fluid in patients, and the drug susceptibility screening confirmed that amphotericin B and caspofungin are effective against *Candida* spp. but some *C. glabrata* and *C. albicans* isolates showed resistance to fluconazole.

Keywords: Bronchoalveolar lavage; *Candida albicans*; *Candida glabrata*; Immunocompromised patient; Fluconazole; Amphotericin B

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INTRODUCTION

Candida infection in the respiratory tract is an important opportunistic disease in immunocompromised and critically ill patients, and recognition and identification of fungal pathogens are critical to patient treatment (1). In recent years, the use of antibiotics, corticosteroids, and immunosuppressive therapy in individuals with organ or stem cell transplants or human immunodeficiency virus (HIV) has produced clinical challenges due to fungal infection/ colonization (2, 3). *Candida* spp. are part of the normal human mycobiome of the epidermis, oral cavity, and the genitourinary, respiratory, and gastrointestinal tracts. A study has shown a close association between host cells and proteins as crucial components of *C. albicans* biofilms (4, 5). They are the usual cause of invasive yeast infections, with the lung being one of the main sites of infection. However, discrimination between colonization and pulmonary infection with *Candida* species should be construed with excessive prudence.

Colonization by *Candida* species in the respiratory tract of susceptible individuals may have a major role in disseminated candidiasis. Delay in diagnosis of accurate and true *Candida* infection can result in high mortality and morbidity rates (1). Bronchoscopy with bronchoalveolar lavage (BAL) is an efficient procedure for the identification of lung infections (6). Histopathology and a small amount of intracellular yeasts can even be used to diagnose pulmonary candidiasis. Appropriate therapy depends on the patient's immune status and underlying disease, the *Candida* spp. involved, and its sensitivity to specific antifungal drugs. *Candida albicans* remains the most prevalent pathogen species of the *Candida* genus, but non-*albicans Candida*, especially *C. glabrata*, shows high virulence potential as well (7). Resistance to the antimycotic drugs used in fungal infections is increasing (8). Fluconazole, amphotericin B, and caspofungin are prominent antifungal agents. The amphotericin B mechanism of action involves binding to fungal membrane ergosterol (9). Caspofungin, an echinocandin, has been shown *in vitro* and *in vivo* fungicidal activation to *Candida* spp., acting via inhibition of (1,3)-β-D- glucan synthase, an important enzyme in the development of the fungal cell wall (10). The azole-based fluconazole is usually used in the treatment of superficial and invasive candidiasis. The purpose of this investigation was to molecular identification of *Candida* spp. isolates in BAL fluid from immunocompromised and critically ill patients and evaluate their sensitivity to medication.

MATERIALS AND METHODS

Patients. Patients which referred to the Shariati Teaching Hospital in Tehran, Iran, were identified from a clinical database and entered into the survey by a simple random sampling procedure, and patient information was collected by questionnaire.

Inclusion criteria. 1) Immunocompromised and critically ill patients were included: immunocompromised condition or weakened immune system group included patients with different form of cancer and were on treatment with chemotherapy, or who have had an organ transplant, such as renal or bone marrow transplant, 2) Patients could be immunocompromised either because of a medical condition or because they received immunosuppressive medications or treatments. The critically ill patients, had underlying disease that caused a wide range of mild to life-threatening conditions that required hospitalization and critical care.

Exclusion criteria. Patients who used antifungal drugs were excluded.

Clinical specimens. Bronchoalveolar lavage (BAL) fluid specimens, were prepared from 161 hospitalized patients with suspected respiratory fungal disease.

Candida species were isolated from bronchoalveolar lavage of immunocompromised and critically ill patients with clinical signs and symptoms (11).

Specimens were mixed with 10% potassium hydroxide, and examined under microscopy (12), and the BAL fluids were cultured on sabouraud dextrose agar with chloramphenicol. BAL fluid specimens were inoculated onto CHROMagar *Candida*, for evaluation of colony count (13), and to distinguish coinfections of *Candida* spp. as well. Isolation of *Candida* spp. from BAL may indicate colonization rather than infection (2).

Molecular identification: DNA extraction of yeasts. One pure colony of yeasts from CHROMagar *Candida* medium was grown on yeast extract peptone

dextrose (YEPD) agar at 37°C for 24-48 h. DNA was purified of yeast cells by the Qiagen DNA tissue kit (Germany) and preserved at -20°C for further processing.

PCR and sequencing of the D1-D2 section. The D1-D2 section of the large-subunit ribo-somal DNA (rDNA) was amplified with NL1 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and NL4 (5´-GGT CCG TGT TTC AAG ACG G-3´) primers (14, 15) with the sub-sequent plan: 98°C for 5 min, 35 cycles of 98°C for 30 s, annealing at 60°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 5 min. The amplicons of PCR products were sequenced by Macrogen (Korea). The results were assessed by the reference nucleotide se-quences using the GenBank database with the BLAST sequence search tool (http://www.ncbi.nlm.nih.gov/BLAST), and the resulting sequences were submitted to the GenBank.

Antifungal drug susceptibility testing using the CLSI reference method. Assessments of susceptibilities to amphotericin B, fluconazole, and caspofungin (Sigma, Germany) were performed by the Clinical and Laboratory Standards Institute (CLSI) (document M27- 4th Edition) A microbroth dilution assay (16). *Candida parapsilosis* 22019 was used as the ref-erence strain, and all tests were performed in duplicate.

Statistical Analysis. Chi-square tests were used to study the existence of a statistically sig-nificant relationship between *Candida* species and the kinds of underlying diseases. Statistical analysis was done with the SPSS version 16 statistical analysis scheme. Statistical significance was specified as a P value <0.05.

RESULTS

Patients. *Candida* spp. were identified in 69 (43%) of the 161 patients, 26 (37.7%) male and 43 (62.3%) female. The demographics, and clinical characteristics, of immunocompromised and critically ill patients with significant history of respiratory disorders and clinical symptoms are presented in (Table 1).

Direct microscopic examination and growth on CHROMagar *Candida* of BAL specimens. Yeast budding forms and pseudohyphae were observed in bronchoalveolar lavage specimens in direct microscopy. Seventy-one positive *Candida* isolates were obtained from 69 BAL fluid specimens. Two patients showed mixed infections/colonization with *C. albicans* and *C. glabrata* on CHROMagar *Candida* (Table 2).

PCR amplification and sequencing of D1-D2 section. The D1-D2 section of the large-subunit rRNA gene amplified with NL1 and NL4 primers produced segments of ~600 bp (Fig. 1). The sequences were evaluated and compared with the reference information in the GenBank database by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). All clinical isolates were specified to species level. Seventy-one samples were positive infection/colonization for *Candida* species including 39 *C. albicans* (55%), 25 *C. glabrata* (35.2%), three *C. krusei (Pichia kudriavzevii)* (4.2%), two *C. dubliniensis* (2.8%), one *C. parapsilosis* (1.4%), and one *C. tropicalis* (1.4%). The results were deposited to the GenBank database under acces-sion numbers MK732377-MK732446.

Antifungal drug susceptibility. Results of susceptibility assays of the 71 *Candida* isolates revealed two isolates of *C. glabrata* (8%) and two isolates of *C. albicans* (5.1%) to be re-sistant to fluconazole. All isolates were susceptible to amphotericin B and caspofungin, with MIC_{90} values for amphotericin B against *C. albicans* and *C. glabrata* of 0.03 µg/ml and 0.25 µg/ml, respectively, and MIC_{90} values of caspofungin against *C. glabrata* of 0.125 µg/ml and *C. albicans* of 0.03 µg/ml. The susceptibility patterns of *Candida* spp. to antifun-gal drugs are presented in (Table 3).

Statistical analysis. A statistically significant relationship between *Candida* species and the kinds of underlying diseases was not observed (P value <0.906).

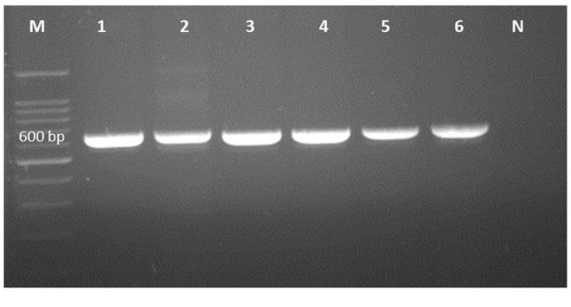

Fig. 1. The D1-D2 region of clinical isolates amplified with NL1 and NL4 primers yielded fragments ~600 bp. Isolates 1, 2: *C. krusei (Pichia kudriavzevii)*; Isolates 3, 4: *C. albicans*; Isolates 5,6: *C. dubliniensis*; M, marker 100 bp; N, negative control.

Table 1. Characteristics of immunocompromised and critically ill patients

Immunocompromised and critically ill patient conditions	n	%	Mean age (range)	Age range	M	F	Clinical symptoms
Lupus	8	11.6	32.5	16-45	8	0	cough, fever, purulent sputum
Diabetes	15	21.7	58.5	25-88	6	9	bloody purulent sputum and chest pain
Heart transplant	2	3	49	42-56	0	2	purulent sputum, cough, asthma
Renal transplant	3	4.3	56.6	33-69	1	2	cough, fever, asthma
Bone marrow transplant	1	1.4	35	35	0	1	fever, purulent sputum,
Liver cirrhosis	5	7.3	63.2	53-75	2	3	bloody purulent sputum, fever
Lung cancer	8	11.6	59.2	32-76	3	5	recurrent fever, purulent sputum
Leukemia	7	10.1	35.7	16-46	1	6	recurrent fever, purulent sputum, chest pain
Renal dialysis	7	10.1	57.3	24-77	2	5	recurrent fever, dyspnea, asthma
Myasthenia gravis	3	4.3	34.3	27-41	2	1	cough, fever, purulent sputum, chest pain
Pneumonia	5	7.3	53.6	37-65	1	4	bloody purulent sputum, cough, fever, asthma
Granulomatosis with polyangiitis (Wegener's granulomatosis)	2	3	49	42-56	0	2	cough, fever, purulent sputum
Atherosclerosis	3	4.3	67.6	54-77	0	3	asthma, cough
Total	69	100			26	43	

Table 2. *Candida* isolates of BAL fluid from immunocompromised and critically ill patients

Patients	n	*C. albicans*	*C. glabrata*	*C. krusei*	*C. dubliniensis*	*C. parapsilosis*	*C. tropicalis*
Lupus	8	5	3	-	-	-	-
Diabetes	15*	8	8	1	-	-	-
Heart transplant	2	2	-	-	-	-	-
Renal transplant	3	2	1	-	-	-	-
Bone marrow transplant	1	1	-	-	-	-	-
Liver cirrhosis	5	4	1	-	-	-	-
Lung cancer	8	4	3	1	-	-	-
Leukemia	7	3	2	-	1	1	-
Renal dialysis	7	3	2	-	1	-	1
Myasthenia gravis	3	2	1	-	-	-	-
Pneumonia	5	2	3	-	-	-	-
Granulomatosis with polyangiitis (Wegener's granulomatosis)	2	1	1	-	-	-	-
Atherosclerosis	3	2	-	1	-	-	-
Total	69	39	25	3	2	1	1

*Coinfection/colonization with *C. albicans* and *C. glabrata* was observed in two diabetic patients.

Table 3. Susceptibility patterns to antifungal agents of *Candida* spp. isolated from bronchoalveolar lavage fluid.

Clinical isolates (n = 71)	Fluconazole			Range: 0.0313-64 µg/ml		Amphotericin B			Range: 0.0313-16 µg/ml		Caspofungin			Range: 0.0313-16 µg/ml	
	S	SDD	R	MIC_{50}	MIC_{90}	S	SDD	R	MIC_{50}	MIC_{90}	S	SDD	R	MIC_{50}	MIC_{90}
C. albicans (n = 39)	37	-	2	0.125	0.5	39	-	-	0.03	0.03	39	-	-	0.03	0.03
C. glabrata (n = 25)	23	-	2	0.125	0.5	25	-	-	0.25	0.25	25	-	-	0.125	0.125
C. krusei (n = 3)	3	-	-	-	-	3	-	-	-	-	3	-	-	-	-
C. dubliniensis (n = 2)	2	-	-	-	-	2	-	-	-	-	2	-	-	-	-
C. parapsilosis (n = 1)	1	-	-	-	-	1	-	-	-	-	1	-	-	-	-
C. tropicalis (n = 1)	1	-	-	-	-	1	-	-	-	-	1	-	-	-	-

Ethical statement. This research was accepted by the Ethics Committee of Iran University of Medical Sciences, under Ethics Committee number IR.IUMS. REC 1396.30578.

DISCUSSION

Respiratory tract fungal infection is the primary reason for morbidity and mortality in immunocompromised and critically ill individuals. Our data were similar to those obtained in previous studies: Delisle et al. (17). investigated *Candida* colonization and its related risk factors to check up clinical results in 639 patients with clinical suspicion of ventilator-related pneumonia and found 17.8% with *Candida* colonization. They reported *C. albicans* in 65.3% of airway specimens, *C. glabrata* in 1.3%, and other non *albicans* spp. in 6.7%. Zarrinfar et al. (12) identified *Candida* spp. in BAL fluid of immunocompromised and immunocompetent patients and observed *C. albicans* to be the most common species in both. Mojtaba Taghizadeh et al. (7) found 31 *C. albicans* (67.39%) and 15 non-*albicans Candida* spp. (32.61%) in BAL fluid.

In the present study, 39 isolates of *C. albicans* (55%) and 32 non-*albicans Candida* spp. (45%) were found. In an autopsy study of cancer patients, *C. albicans* and *C. glabrata* were the most abundant species (18). Pulmonary fungal infection is an important event in seriously ill immunocompetent patients. In a study of immunocompetent patients, the most frequent fungal species reported were of the genera *Aspergillus* (51.4%), *Cryptococcus* (22.9%), and *Mucor* (2.4%) (19).

The emergence of resistant *Candida* spp. such as *C. glabrata, C. krusei (Pichia kudriavzevii),* and *C. albicans* due to the use of azoles as prophylaxis for fungal infections makes recognition of *Candida* to the species level crucial for appropriate treatment, and it is very important that *C. albicans* and *C. glabrata* are a highly adaptable microorganisms, being able to develop resistance by over expression of efflux pumps following prolonged exposure to antifungals, toxic substances and metabolites (20). However the agents of drug resistance in fungi are multifactorial.

We determined 5.1% resistance of *C. albican*s to fluconazole and found no resistance to amphotricin B or caspofungin. Taghizadeh et al. (7). reported widespread resistance to ketoconazole, clotrimazole, and fluconazole, but significant resistance (8.69%) was observed only to fluconazole, and most isolates of *Candida* spp., were sensitive to ketoconazole, clotrimazole, fluconazole, and nystatin. Biernasiuk et al. (21). analyzed drug susceptibility of *C. albicans* isolated from the respiratory tract of patients with chronic hepatitis C who had not used antiviral drugs, and from those who received peginterferon and ribavirin, to amphotericin B, fluconazole, flucytosine and miconazole reported 100% of *C. albicans* isolates sensitive to amphotericin B and flucytosine. *Candida albicans, C. tropicalis, C. glabrata,* and *C. krusei* infected isolates from various sites of immunocompromised patients were found resistant to amphotericin B; whereas, in colonized isolates, resistance to amphotericin B was seen only in *C. albicans* and *C. krusei* and, to fluconazole, only in *C. albicans* (0.5%) and *C. tropicalis* (8.8%) (22). Castanheira et al. (23). found 0.2% of 1310 *C. albicans* isolates resistant to caspofungin, 0.2% showing micafungin resistance, and 0.4% resistant to fluconazole. They reported a MIC_{90} value for amphotericin B of 1 μg/ml for all *Candida* isolates, except for a 2 μg/ml MIC_{90} value for *C. krusei*. *Candida glabrata* isolates revealed the highest echinocandin resistance rates to micafungin, anidulafungin, and caspofungin, and 8% of *C. glabrata* isolates were resistant to fluconazole. Taghipour et al. reported *C. albicans* and *C. glabrata* were important isolates of respiratory tract secretions (24). In the first multicenter study, in non-neutropenic, critically ill adult patients, *C. albicans* was the most common species followed by *C. glabrata* in invasive fungal diseases (25). Fracchiolla et al. reported *Candida* spp. such as *C. albicans, C. glabrata,* and *C. krusei* isolated from bronchoalveolar lavage, of oncohematological patients (26). In this study, a statistically significant relationship between *Candida* species and kinds of underlying diseases was not observed. Therefore we can focus on particular underlying diseases and *Candida* species in further study. We found all *Candida* isolates to be susceptible to caspofungin and amphotericin B. Consideration of resistance to fluconazole of *C. glabrata* and *C. albicans* isolates is necessary for effective patient treatment.

CONCLUSION

Our study showed that *Candida albicans* and *C.*

glabrata are the most commonly isolates of immunocompromised and critically ill patients, and the drug susceptibility screening confirmed that amphotericin B and caspofungin are effective against *Candida* species but some isolates of *C. albicans* and *C. glabrata* were resistant to fluconazole. The limitations of this study were the absence of tissue diagnosis, according to "EORTC/MSG 2020". As tissue sampling is an invasive diagnosis method, the specialists prefer not to perform it in high-risk patients. Therefore, the diagnosis was performed based on observation of yeast and pseudohyphae in BAL samples. Furthermore, the limited access to the immunocompromised patients willing to participate in the study resulted in the small sample size.

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