

# Molecular Identification and Evaluation of Drug Susceptibility of *Candida* Isolates from Bronchoalveolar Lavage Fluid in Patients with Hematological Malignancies

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## Abstract

**Background:** Different studies have shown that despite the expanding number of antifungal drugs, the death rate caused by *Candida* species has increased during the recent decades due to drug resistance occurrence.

**Objectives:** The present study aims to identify molecular structure and evaluate drug susceptibility in *Candida* species isolated from bronchoalveolar lavage fluid in patients diagnosed with hematological malignancies.

**Methods:** In this cross-sectional study, 54 clinical specimens were taken from the bronchoalveolar lavage of patients. The suspected colonies were investigated by microscopic examination and subsequent passages were evaluated according to standard operating procedures and specification of the type of colony color prescribed by CHROMagar to isolate the yeast. The sequencing method (ITS1, ITS4) was used to approve *Candida* species. Finally, susceptibility test was carried out according to M27S-3 and M38-A2 micro-dilution methods.

**Results:** Among 54 samples investigated with culture and PCR methods, 33 *Candida* species were identified in patients with hematological malignancies. *Candida albicans* (75.7%) was the most common fungal isolate. Results of drug susceptibility tests showed that the isolated *C. albicans* (n = 2), *C. glabrata* (n = 1), and *C. tropicalis* (n = 1) from patients with hematological malignancies were resistant to fluconazole. The present study showed that the prevalence of *C. albicans* was higher than other fungal species among patients with hematological malignancies.

**Conclusion:** *Candida* species are more susceptible to voriconazole, amphotericin B and Caspofungin. Therefore, identification of *candida* species along with their antifungal susceptibility pattern can help clinicians to better treat patients.

**Keywords:** Fungal, Drug Susceptibility, Hematological Malignancies, Patient

## 1. Background

*Candida* species are a major component of normal skin and mucosal surfaces flora.<sup>1</sup> In an immunodeficient host they can lead to superficial, cutaneous, mucosal or systemic opportunistic fungal infections.<sup>2</sup> *Candida* infections are among the most important and common opportunistic fungal infections that can occur in acute, severe and chronic forms in skin, lung, vagina and gastrointestinal tract.<sup>3</sup> Immunosuppressive factors such as chemotherapy and radiation therapy in cancer patients, broad-spectrum antibiotics, HIV infection, and diabetes mellitus can also aggravate these fungi.<sup>4</sup> Invasive candidiasis is a common and potentially fatal complication caused by cancer and related chemotherapy.<sup>5</sup> Most of *Candida* infections are caused by *Candida albicans* (*C.*

*albicans*) where in almost half of the cancer patients undergoing head and neck radiation therapy, the *Candida* species accumulates and lead to infection, which could be fatal if the infection is developed locally or systemically.<sup>6</sup> Although the use of novel antifungal drugs has been advantageous in the control of fungal infections, the main problem in early diagnosis of the infection agent remains.<sup>7</sup> Therefore, early onset of treatment is an important criterion in reducing the mortality rate in immunodeficient patients.<sup>8</sup>

Respiratory tract is the most common place for fungal accumulation and onset of infections in immunodeficient patients including cancer patients receiving immunosuppressive drugs, and is also associated with high mortality rate in this group.<sup>9</sup> In most cases, isolation and

identification of fungal agents from respiratory tract secretions are the first diagnostic step in this group of patients that can be detected in several different forms such as presence of colonized fungus. Candidemia is associated with excess morbidity and mortality, longer hospital stays, and higher hospital costs, especially in severely immunosuppressed patients with hematologic malignancies. Occasionally, fungal accumulation may be caused by small or general pulmonary malformations or lesions; while cortisone therapy, chemotherapy and radiotherapy can also enhance fungal colonization in hematologic malignancies patients.<sup>10</sup> Studies on the hospitalized patients in the intensive care units in different countries have shown that a significant percentage of the occurring pathogens were fungal, dominated by *Candida* species followed by *Aspergillus*.<sup>11</sup>

Polymerase Chain Reaction (PCR) is used as a complete, simple, specific, sensitive and reliable method of *Candida* species detection and identification. Amplification of nucleic acid sequence and sequencing of rDNA coding genes are still implemented as the gold standard for identification. Drugs used to treat candidiasis are limited, while the sensitivity of *Candida* isolates to antifungal drugs, especially azoles, is decreasing.<sup>12</sup> Nowadays, by increased consumption of Amphotericin B and azoles in the treatment of invasive fungi, drug resistance and reduced sensitivity of some of the azoles has become prevalent and caused problems in the treatment procedures.<sup>13</sup> Therefore, determination of sensitivity pattern to antifungal drugs and identification of emergence of resistance is of significant importance, especially since resistance to drugs is the most important challenge in the treatment of these patients.

In recent years, to their resistance to some antifungal drugs, non-*albicans* *Candida* such as *C. tropicalis* and *C. glabrata* have been subject of more research. With the increasing resistance of *Candida* isolates to antifungal drugs, assessment of drug sensitivity of isolates from at-risk patients may help to improve the treatment of these patients.<sup>14,15</sup>

## 2. Objectives

With respect to the importance of identification of *Candida* species isolates, especially those with less sensitivity to antifungal drugs in high-risk patients such as cancer patients undergoing chemotherapy, the present study was aimed to identify the isolates from Bronchoalveolar Lavage (BAL) fluid of hematological malignancies patients using molecular methods and evaluated the drug sensitivity of these isolates under CLSI standards.

## 3. Methods

### 3.1. Sampling

This descriptive cross-sectional study was carried out over a period of one year, from June 2020 to June 2021 in

Shariati teaching hospital, Tehran province, Iran. The inclusion criteria in this study consisted of the three following conditions:

1) Patients with diagnosed hematological malignancies and presenting clinical symptoms of pulmonary fungal infections (dyspnea, cough, high and persistent fever, recurrent fever, chest pain, purulent sputum, weight loss, night fever, hemoptysis, rhinitis, and wheezing) based on the professional opinion of a pulmonary disease specialist.

2) Patients who had not taken any systemic antifungal drugs before enrollment in treatment procedures to prevent any occurrence of false-negative results.

3) Patients who are not lost for follow-up and were not deceased during the study period.

Clinical history of the patients including demographic features (age, gender) and precursor diseases as well as clinical manifestations of symptoms (fever, cough, dyspnea and sputum) were recorded. The BAL samples were obtained from all participating patients.

After collection, the specimens were swiftly transported to the laboratory to perform all microscopic and culture experiments on the specimens in a one-hour period. BAL samples were centrifuged and the deposit was used for examination. In the direct microscopic examination, the samples were first dissolved in KOH 10% solution on microscopic slide and then investigated under a microscope for fungal elements and all specimens were cultured on Sabouraud Dextrose Agar (SDA) (Merck, Germany).

CHROMagar *Candida*, was used for colony count and assessment of co-infections of *Candida* spp as well. Any growth obtained was further inspected for its growth rate, colony morphology, and lacto phenol cotton blue mounts. All isolates of *Candida* spp were further subjected to PCR and sequencing for molecular speculations.

### 3.2. Polymerase Chain Reaction

Fungal genomic DNA was extracted from harvested colonies using phenol-chloroform method. The internal transcribed spacer ITS region was amplified and sequenced using primers ITS1 (5'-TCCGTAGGTGAAC CTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3'), which have been previously described.<sup>9,11</sup> Briefly, the amplification of ITS region was performed for 5 min at 94 °C for primary denaturation, followed by 35 cycles at 94 °C (30 seconds), annealing at 56 °C (30 seconds), and 72 °C (30 seconds), with a final 7 min extension step at 72 °C. PCR products were checked in 1.2% agarose gels with DNA safe stain and visualized with UV. The PCR products were then sent for sequencing and DNA sequencing results were compared with the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>).

### 3.3. Antifungal Drug Susceptibility Test

All *Candida* species isolates were tested regarding the

Clinical and Laboratory Standards Institute (CLSI) document M27-S3 and S4. The four antifungal agents included in this study were Fluconazole (FLU), Voriconazole (VOR), Caspofungin (CAS), and Amphotericin B (AmB).

Powders of AmB (Sigma, Germany), FLU, VOR, and CAS (Sigma, USA) were obtained from the respective manufacturers. RPMI 1640 (Sigma, St. Louis, Missouri) was made according to the manufacturer’s protocol and buffered to pH 7.0 with 0.165 mol/L (MOPS) [3-(N-morpholino) propane sulfonic acid] buffer (Sigma, USA). Stock solutions with a 10-fold concentration were prepared in dimethyl sulfoxide (DMSO) or water for each antifungal agent. Supplementing the necessary amount of PRMI medium achieved the final concentrations of the working solutions. The final concentrations of the antifungal agents were 0.0313 to 16 µg/ml for AmB and VOR, 0.125 to 32 µg/ml for FLU, and CAS. The inoculum suspensions (0.5 McFarland) were prepared by the spectrophotometric method at 530 nm; optical density: 75-77 for *Candida* spp, (Pharmacia biotech Cambridge, England ultrospec 3000 UV/visible spectrophotometer). A 100-µl volume of fungi inoculum and an equal volume of antifungal agents were added to each well. Drug-free and fungi-free wells were included as positive and negative controls. The Minimum Inhibitory Concentration (MIC) of AmB was reported as the lowest drug concentration with complete inhibition of any discernible growth (100%) and for FLU, VOR, and CAS the lowest concentration that inhibits the growth, compared to positive controls.<sup>16</sup>

**3.4. Statistical Analysis**

The data analysis was performed using SPSS software (IBM SPSS Statistics for Windows, Version 22.0, IBM Corp). The study was assessed by standard chi-square and t-test (95% confidence intervals). The *P*<0.05 was considered as statically significant.

**Table 1.** Characteristics of Patients

Patient characteristics		Frequency %	* <i>p</i> value
Sex	Male	36 (66.7)	0.014
	Female	18 (33.3)	
Age	<40	33 (61.1)	0.102
	>40	21 (38.9)	
Symptoms	Fever	37 (68.5)	0.022
	Cough	28 (51.8)	
	Dyspnea	18 (33.3)	
	Sputum	40 (74.1)	
Antifungal therapy	Yes	0	
	No	54 (100)	

\**p*<0.05 indicated significant difference between two groups

**Table 2.** Identification of Clinical Isolates of *Candida* species using Conventional Methods

Species	CHROMagar	Frequency %
<i>C. albicans</i>	Light green	25 (75.7)
<i>C. glabrata</i>	Pink	1 (3.05)
<i>C. tropicalis</i>	blue	6 (18.2)
<i>C. inconspicua</i>	Light Pink	1 (3.05)

**4. Results**

**4.1. Patient Demographics**

A total of 54 hematological malignancy patients were included in the present study. Patient cohort had a mean of 48.4 years old. The characteristics information ihas been shown in Table 1. Patients displayed similar median leukocyte counts and CRP in the initial tests (*P*>0.05). Duration of hospitalization was 14.33 ± 6.82 days for the study group. The majority of patients were displaying fever and sputum and required bronchoscopy. Neither of the patients were on antifungal therapy at the time of hospitalization.

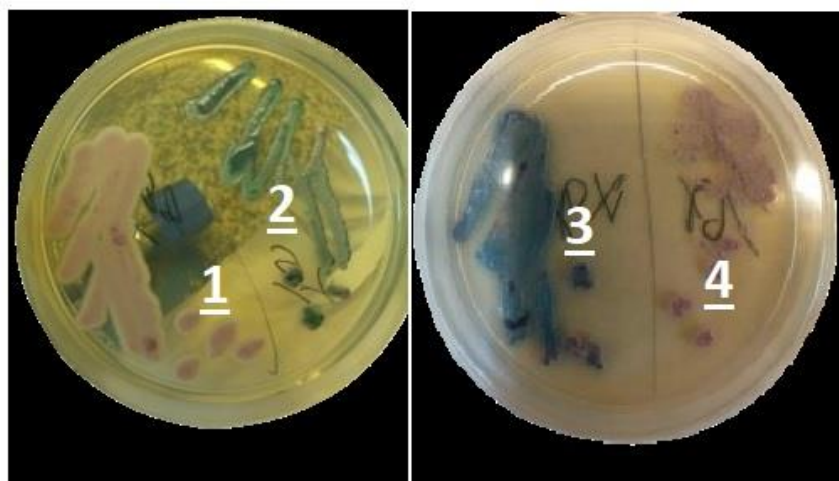
**4.2. Morphologic and Molecular Identification**

Conventional and molecular assays were utilized for the identification of 33 *Candida* isolates obtained from 54 hematological malignancy patients (Table 2). The colonies were identified by colony color and the identifications were confirmed using the PCR method. The isolated *Candida* species and their expected color according to manufacturers have been presented in Figure 1.

The ITS1 and ITS4 was successfully amplified in all *Candida* isolates. All of the sequences had been deposited in GenBank under the accession number reported in Table 3. The PCR results for identifying different species of *Candida* have been summarized in Table 3. *C. albicans* (75.7%) was the most commonly occurring species among the 33 obtained *Candida* isolates in this study (Figure 2).

**4.3. Antifungal Susceptibility**

The MIC of drugs against *Candida* strains were determined to be within acceptable ranges proposed by CLSI guide line and *Candida parapsilosis* 2019 was used as the reference strain. MIC ranges have been presented in Table 4 along with geometric MIC, MIC<sub>50</sub>, and MIC<sub>90</sub>. However, MIC<sub>90</sub> was not measured when fewer than nine

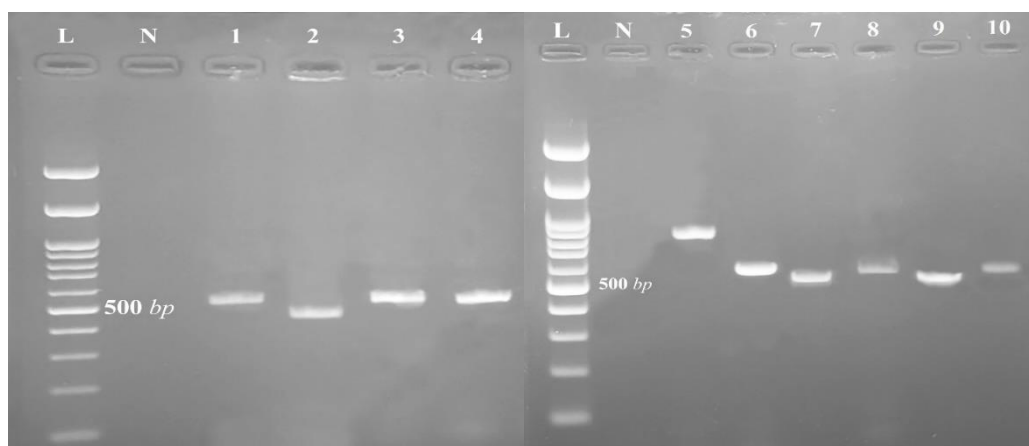


**Figure 1.** Color of the Colonies on CHROMagar *Candida* spp. The colonies of *C. inconspicua*, *C. albicans*, *C. tropicalis* and *C. glabrata* are marked with numbers 1 to 4 respectively

**Table 3.** Primer Pairs used to Identify *Candida* spp

<i>Candida</i> spp.	GenBank accession no.	Size of DNA (bp)	Primer sequence (5' to 3')
<i>C. albicans</i>	LC522908	529-535	
<i>C. tropicalis</i>	MT169776	869-879	F: TCCGTAGGTGAACCTGCGG
<i>C. glabrata</i>	LC317498, FN652301	517-524	R: TCCTCCGCTTATTGATATGC
<i>C. inconspicua</i>	AY936515	447-449	

F: Forward, R: Reverse.



**Figure 2.** Agarose Gel Electrophoresis Pattern of Polymerase Chain Reaction Products of ITS Region of *C. albicans* (529-535 bp): 1, 3, 4, 6, 8, and 10; *C. glabrata* (869-879 bp): 5; *C. tropicalis* (517-524 bp): 7 and 9; *C. inconspicua* (447-449): 2. L: Ladder; N: Negative control.

**Table 4.** Susceptibility Criteria to Antifungal Drugs of *Candida* spp

Species	Antifungal	Frequency		MIC <sub>50</sub> (µg/ml)	MIC <sub>90</sub> (µg/ml)	Range (µg/ml)
		Sensitive	Resistant			
<i>C. albicans</i>	Fluconazole	23	2	0.5	1	0.124-64
	Voriconazole	25	-	0.031	0.125	0.031-16
	Amphotericin B	25	-	0.031	0.062	0.031-16
	Caspofungin	25	-	0.015	0.125	0.015-8
<i>C. glabrata</i>	Fluconazole	-	1	-	-	0.124-64
	Voriconazole	1	-	-	-	0.031-16
	Amphotericin B	1	-	-	-	0.031-16
	Caspofungin	1	-	-	-	0.015-8
<i>C. tropicalis</i>	Fluconazole	5	1	-	-	0.124-64
	Voriconazole	6	-	-	-	0.031-16
	Amphotericin B	6	-	-	-	0.031-16
	Caspofungin	6	-	-	-	0.015-8
<i>C. inconspicua</i>	Fluconazole	1	-	-	-	0.124-64
	Voriconazole	1	-	-	-	0.031-16
	Amphotericin B	1	-	-	-	0.031-16
	Caspofungin	1	-	-	-	0.015-8

MIC: The minimum inhibitory concentration.

isolates were available.

*C. albicans*, the most prevalent isolated species, were sensitive to VOR, AmB and CAS with 0.031, 0.031, and 0.015 MIC<sub>50</sub>, respectively (Table 4). The results show that 8.7% (2 samples) of *C. albicans* isolated from patients were resistant to FIU. The isolate of *C. glabrata* was resistant to fluconazole, although this isolate was sensitive to AmB, VOR, and CAS. Among the five *C. tropicalis* isolates, four samples (80%) were found to be sensitive to FLU and, five isolates of *C. tropicalis* (100%) were sensitive to AmB, VOR and CAS (Table 4).

## 5. Discussion

Studies on hospitalized patients in different countries showed that a significant percentage of pathogens were fungi related, most commonly reported being *Candida* species.<sup>17</sup> In the present study, out of 54 samples collected from patients with hematologic malignancy, 33 cases (61%) were diagnosed with pulmonary fungal infection. PCR revealed that *Candida albicans* was the most common fungal infection in hematological malignancies patients with a frequency of 75.7%, which was consistent with previous reports.<sup>18</sup>

A study by Badiie et al.<sup>19</sup> in 2016 also showed that *C. albicans*, *C. glabrata* and *C. krusei* had the highest frequency in patients with pulmonary malignancies, which was consistent with this study. In the present study, the PCR technique was utilized to show that *C. Albicans* and *C. tropicalis* and were the most dominant fungal infections in hematological malignancies patients, with 75.7% and 18.2% frequencies respectively.

In a 2018 study by Kianipour et al.,<sup>20</sup> 59 yeast samples were identified among 120 BAL samples collected, with 29 total *Candida* samples consisting of 17 samples of *C. albicans* and *C. dubliniensis* species (58.6%) and 12 samples of non-*albicans* *Candida* (41.4%). Yang et al.<sup>21</sup> also reported the dominant species in their study as *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. dubliniensis* with frequencies of 62.1%, 17.9%, 8.3% and 2.7%, respectively. Abundance of reports on the invasiveness of non-*albicans* species in cancer patients amplify the importance of using highly accurate diagnostic methods such as PCR.

Due to the increased prescription of antifungal drugs such as fluconazole, voriconazole, caspofungin and amphotericin B in fungal disease treatments, we faced increased resistance and reduced sensitivity of some fungal isolates against these drugs. Khodavaisy et al.<sup>22</sup> in 2020 reported reduced susceptibility of non-*albicans* *Candida* species to itraconazole. Shokouhi et al.<sup>23</sup> also showed that 2.6% of *Candida* species were resistant to fluconazole and amphotericin B and also 5.4% of them were resistant to itraconazole, but all species were sensitive to caspofungin which is consistent with the present study. In the present study, MIC as a susceptibility gauge of

*Candida albicans* isolates to antifungal drugs were calculated for fluconazole, voriconazole, amphotericin B and caspofungin and were 0.5, 0.031, 0.031 and 0.015, respectively. The highlight of our study was that all isolates of *C. glabrata* and one isolate of *C. tropicalis* were resistant to fluconazole, indicating an increase in resistance of non-*albicans* species to fluconazole, which could be a serious threat to the treatment of fungal infections in hematological malignancy patients.

Long-term prophylaxis with fluconazole in patients under treatment can be one of the causes of resistance, so physicians should be cautious in the use of azoles in their treatments. All *Candida* isolates in our study were sensitive to voriconazole, amphotericin B and caspofungin, so voriconazole can be mentioned as the drug of choice in the treatment of fluconazole resistant species that needs further investigation.

## 6. Conclusion

The present study showed that the prevalence of *Candida albicans* was higher among patients with hematological malignancies compared to other *Candida* species. *Candida* species are more sensitive to voriconazole, amphotericin B and caspofungin, so identifying *Candida* infections along with their antifungal susceptibility pattern can assist physicians in better treatment of patients. Results of this study can be incorporated into a better and more effective selection of treatment method as well as to promote the communities' health.

### Research Highlights

#### What Is Already Known?

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.

#### What Does This Study Add?

The result of this study showed that the prevalence of *Candida albicans* was higher among patients with hematological malignancies compared to other *Candida* species. *Candida* species are more sensitive to voriconazole, amphotericin B, and caspofungin.

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### Author Contributions

Conceptualization: ShF; Data curation: TE, FK, and RA; Formal Analysis: TE, FK; Funding acquisition: ShF; Investigation: FK, ZG, MD, and MF; Methodology: FK,

TE, RA, and ZGh; Project administration: ShF and MF; Software: ShF, TE, and MD; Supervision: MF, ZGh; Validation: ShF; Writing original draft: FKh, TE; Writing review & editing: ShF, MD, and RA.

### Conflict of Interest Disclosures

All authors declared that they have no conflict of interest.

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