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ORIGINAL ARTICLE

Distribution of fungal agents in the respiratory system of patients with underlying lung diseases; molecular identification and antifungal susceptibility profiles

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ABSTRACT

Background and Objectives: Airway fungal infection is a severe clinical problem, especially in patients with compromised immune functions. Here, we examined the distribution and antifungal susceptibility profiles of fungal agents isolated from respiratory tract of symptomatic patients hospitalized in pulmonary units.

Materials and Methods: This descriptive cross-sectional study took place from 2023 to 2024, involving 360 patients. Bronchoalveolar lavage (BAL) or sputum specimens were collected and analyzed using mycological and molecular methods for this study. Antifungal susceptibility testing (AFST) was carried out using the broth micro dilution method.

Results: Of a total of 360 respiratory specimens, 114 (31.6%) were positive. The male-to-female ratio was 63:51 (1.3%). *Candida albicans* and *Aspergillus flavus* were the most common yeast and mold species. Chronic obstructive pulmonary disease (COPD) had the highest rate of colonization with fungal agents (47/114, 41%). The isolates associated with COPD in this study included *Aspergillus* species (4/12, 3.5%), *Candida* species (41/96, 36%), and other fungal species (2/6, 1.5%). Coughing (87%) was the predominant symptom, and malignancy (52%) was the predominant comorbidity factor. The result of AFST for antifungal agents showed that 9 (22.5%) *Candida* isolates were resistant, and the highest rate of resistance was related to voriconazole agent (5/9, 55.5%). Resistance to antifungal agents was not observed among *Aspergillus* isolates.

Conclusion: This study showed a significant relationship between the frequency of *Aspergillus* and *Candida* species in patients with underlying lung diseases. In addition, voriconazole was more effective than itraconazole, especially against *Aspergillus flavus.*

Keywords: Lung infection; Underlying lung diseases; Antifungal; Fungal agents; Polymerase chain reaction; Iran

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INTRODUCTION

Fungal lung infections have emerged as a worldwide healthcare problem in the last two decades, a significant proportion of which are community-acquired (1). Respiratory system infections occur following exposure to yeasts and molds implicated in lung pathologies. Clinical features, disease severity, and mortality rates vary among patients with underlying lung disease or individuals exhibiting symptoms such as cough, shortness of breath, chest pain, and bronchiectasis (2). Diagnosing fungal respiratory infections often relies on a combination of clinical, radiologic, and microbiological factors (3). The bronchoalveolar lavage (BAL) specimen represents a valuable tool for diagnosing fungal lung infections in high-risk patients, and sputum culture provides diagnosis in less than 50% of pneumonia patients (4, 5). Early and precise diagnosis of fungal lung infections is crucial for timely starting antifungal treatment and reducing the unnecessary use of toxic antifungal agents. Although traditional approaches such as direct microscopic examination, histopathological evaluation and cultivation are still the gold standard, of PCR-based diagnosis assays have been developed, and they can improve early diagnosis of fungal lung infections with the advantages of high sensitivity, the ability to establish diagnosis at the species level, and the capacity to detect genes that confer antifungal resistance. Several studies have indicated that most respiratory infections stem from the genera *Aspergillus* and *Candida* (6). To our knowledge, susceptibility assessments for fungi are not routinely performed in treating pulmonary infections. Therefore, clinicians must be familiar with clinical presentation, diagnostic methods, and treatment management. In this study, we investigated the distribution of fungal agents in the respiratory system among patients with underlying lung disease using molecular analysis and phylogenetic tools. Additionally, we examined the antifungal susceptibility profiles of isolated strains using the broth microdilution method. This study emphasized the microbiological and epidemiological aspects of the topic.

MATERIALS AND METHODS

Study design and dligibility criteria. This descriptive cross-sectional study was carried out from 2023 to 2024, involving 360 individuals previously diagnosed with underlying lung disease and hospitalized in different centers in Tabriz, Iran. This study was approved by the ethical committee of Tehran University of Medical Sciences (the number of Ethics Committee protocol: IR. TUMS. SPH.REC.1402.106). All subjects or their companions were provided with written informed consent before sample collection. Subsequently, all data were collected. Additionally, medical records, including information on underlying lung disease, comorbidity impact, clinical symptoms, radiographic signs, as well as demographic data such as age and sex, were captured and documented whenever available. The criteria for entering people into this study included patients who were previously diagnosed with an underlying lung disease (Chronic obstructive pulmonary disease) (COPD), asthma, bronchiectasis, pulmonary tuberculosis (TB), interstitial lung disease (ILD), and others, who were hospitalized. Exclusion from the study was applied to patients who had received systemic antifungal treatment within three days preceding sample collection.

Specimens collection and mycological examination. Sputum specimens and BAL specimens were collected by Physicians and immediately sent to the laboratory of medical mycology at the School of Medicine in Tabriz, and microscopic and culture experiments were performed on the specimens in the shortest time possible. Before microscopic examination and performing culture cultivation, the sputum samples were diluted with sterile saline, BAL samples were centrifuged and the sediment of this sample was used for analysis. All sputum and BAL samples were examined using potassium hydroxide (KOH 10%) solution and examined under a microscope (Olympus. Germany) to detect presence or absence of the fungal agents. Culturing involved inoculating samples on Sabouraud dextrose agar (SDA) (Merck, Germany) supplemented with antibiotics (chloramphenicol) and Brain Heart Infusion (BHI) agar media (Merck, Germany), followed by incubation at 32°C for 72 hours (7). After colony growth, the isolates were identified by macroscopic appearance (hypha/yeast, Surface topography, texture, and pigment). Yeast isolates were identified based on mycological tests including production of chlamydoconidia in cornmeal agar (Becton, France) and colony color on chromogenic CHROM agar *Candida* medium (HiCrome™, France). Filamentous fungi were recognized using morphological characterization on Czapek Dox Agar

(Sigma Aldrich, USA) and slide culture technique. Eventually, for molecular assays and antifungal susceptibility testing (AFST), we transferred pure colonies to eppendorf tubes containing sterile water and stored them at -18°C (8).

Molecular identification of fungal isolates: Genomic DNA extraction. DNA was extracted from the mycelium of pure culture colonies using phenol-chloroform method (9), and the boiling method was applied for DNA extraction of the yeasts (10). DNA quality was checked via running 2-3 µL on an agarose gel (0.8%). In order to determine yeast, the primers were used for the amplification of the internal transcribed spacer (ITS) region (ITS 1, 5′ -TCC GTA GGT GAA CCT GCG G-3′; ITS 4, 5′ -TCC TCC GCT TAT TGA TAT G-3′) (8), and in order to determine *Aspergillus*, tubulin gene (benA), was amplified and sequenced using *Bt2a* (5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′) and *Bt2b* (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (11).

Polymerase chain reaction (PCR) and sequencing analysis. Polymerase chain reaction (PCR) amplification for each *Aspergillus* isolate was performed as described previously (8). Positive PCR products were sent for sequencing (Codon genetics group, Iran). The sequences were aligned using the Basic Local Alignment Tool Search (BLAST) to check their probable similarities with submitted fungal sequences on GeneBank.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A PCR-RFLP method for identification *Candida* spp. was carried out according to the previously approved protocol introduced by Shokohi et al. (12). In this procedure we used MspI (*HpaII*) enzyme. Finally, *Candida* spp. were identified according to each electrophoretic band pattern and size.

***In-vitro* antifungal susceptibility testing (AFST).** The AFST was performed using broth micro dilution techniques according to Clinical and Laboratory Standards Institute guidelines (CLSI), M27-A3, S4 and M38-A2, M59 (13-15). In brief, the susceptibilities of the isolates to itraconazole (ITC) (Sigma-Aldrich, USA), voriconazole (VRC) (Sigma-Aldrich, USA), amphotericin B (AMB) (Sigma-Aldrich, USA), and caspofungin (CAS) (Sigma-Aldrich, USA), were assayed by the broth micro dilution method. Isolates were grown on potato dextrose agar (PDA) at 35°C up to 48-72 h to maximize conidial harvest, and the conidia were counted with a Neubauer chamber and adjusted to a concentration of 10^6 CFU/mL (13, 16). The micro dilution was performed with twofold dilutions of the drugs at concentrations ranging from 0.031 to 16 µg/mL. For *Candida* and *Aspergillus* isolates, the Minimum inhibitory concentration (MICs) endpoints for AMB, ITC, VRC and CAS were determined using a reading mirror as the lowest concentration of the drug at which it prevents any recognizable growth (100% inhibition) (8, 15). Minimum effective concentration (MECs) were determined for CAS in the *Aspergillus* isolates and they were only defined microscopically. At the lowest concentrations, the drug leads to the growth of small, rounded, compact hyphal forms as opposed to the long, unbranched hyphal clusters seen in the growth control. For quality control, strains including *Aspergillus flavus* ATCC®204304 and *Candida parapsilosis* ATCC®22019 were incorporated, relying on their defined MICs. Susceptibility assessments were conducted through duplicate measures in laboratories. We used the previously described Epidemiological cut-off values (ECVs) for each antifungal to detect the isolates within each species that might have acquired a mutational resistance mechanism to a given agent (17). Also for *Aspergillus* isolates, according to CLSI guideline we used terms wild-type (WT) and non-wild–type (NWT) instead of susceptible and resistant, respectively. We used AFST just for *Candida* and *Aspergillus* isolates.

Statistical analysis. The collected data were analyzed in SPSS software (version 26, Statistical Product and Services Solutions, Inc, Chicago, IL, USA), and a p-value of ≤ 0.05 was considered statistically significant. We summarized the data using descriptive statistics, presenting continuous variables as median, interquartile range, and categorical variables as proportions or percentages.

RESULTS

Study population and clinical profile of patients. A total of 360 patient cases, hospitalized in different centers, 206 (57%) male and 154 (43%) female were recruited. The male to female odds-ratio was 206:154 (1.33%). Of this population, 114 (31.6%) were pos-

itive that the male-to-female ratio was 63 (55.2%) to 51 (44.8%). According to the age of the patients, 25 (7%) of the patients were less than 10 years old, 220 (61%) of the patients were between 11-49 years old, and 115 (32%) of the patients were over 50 years old. All patients were previously diagnosed with an underlying lung disease and hospitalized in different centers in Tabriz, Iran (Table 1). In this study, the average length of a patients' hospital stay was 14 days and the length range was 1 to 45 days. In the pulmonary function tests conducted by a specialist doctor, most patients showed COPD (41%) and asthma (21%) followed by shortness of breath, hemoptysis, chest pain, and high fever (Fig. 1). In the review of radiological findings performed on patients on average one week after the onset of symptoms, the main findings were lobular infiltration and multifocal infiltration, and more specific symptoms, such as halo or air crescent symptoms, were not observed. The recorded data of patients' files showed that diabetes mellitus (DM), cardiovascular disease (CVD), renal disease, malignancy, and autoimmune disease had comorbidity impacts in hospitalized patients. Demographic and clinical information of pulmonary patients is represented in Fig. 2.

Fungal agents and correlation with underlying lung diseases. The investigated samples were collected from patients by sputum 76 (21%) and BAL 284 (79%), and the results showed that 114 fungal species were isolated from 360 investigated patients, and the frequency of isolates was 31%. Among the study population, the specimen's frequency according to gender was 41 (54.2%) sputum specimens and 164 (57.7%) BAL specimens were collected from males. Meanwhile, 35 (45.8%) sputum specimens and 120

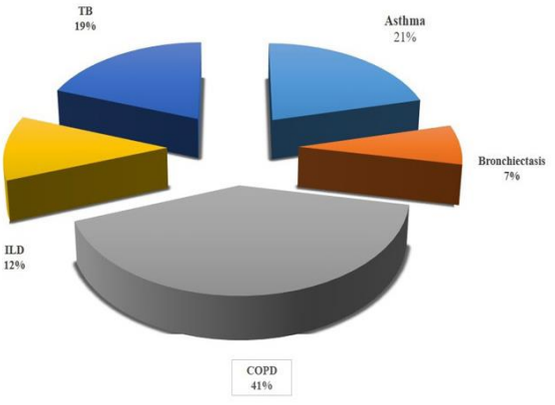

Fig. 1. Frequency of underlying lung diseases in hospitalized patients (N %).
COPD, Chronic obstructive pulmonary disease; ILD, interstitial lung disease; TB, tuberculosis

(42.3%) BAL specimens were collected from females. Direct microscopic examination showed the presence of hyphae in 3 patients and budding yeast cells in 10 patients; these isolates also were isolated by culture. Finally, 114 isolates were observed by culture and microscopic examination. This result means that direct microscopic examination did not identify 101 out of 114 isolated fungi observed by culture (Table 2). The results showed *Aspergillus* spp. presence in 12 patients (including *A. flavus* in 6 patients, *A. fumigatus* in 2 patients, *A. terreus* in 1, *A. tubingensis* in 1, and *A. niger* in 2 patients) and *Candida* spp. presence in 96 (*C. albicans, C. tropicalis, C. krusei, C. parapsilosis* and *C. glabrata* being isolated from 56, 17, 9, 6 and 8 patients, respectively). Six patients with different underlying lung diseases showed growth of other fungal isolates. The distribution of the fungal isolates concerning the diagnostic groups is represented in

Table 1. Socio-demographic and clinical data of patients who participated in the study

Variables	Total 360 (100%)	Colonization 114 (100%)	p-Value	Odds Ratio (OR) 95% confidence interval (95% CI)
Gender				
Male	206 (57%)	63 (55%)	0.86	OR:1.57
Female	154 (43%)	51 (45%)		95% CI: (1.52 to 1.62)
Age groups (years)				
<10	25 (6.9%)	1 (0.8%)	0.57	OR:2.24
11-49	221 (61%)	65 (57%)		95% CI: (2.18 to 2 .30)
≥50	114 (32.1 %)	49 (42.2%)		
Specimens				
Sputum	76 (21%)	26 (23%)	0.00	OR:1.23
Bronchoalveolar Lavage	284 (79%)	88 (77%)		95% CI: (1.19 to 1.28)

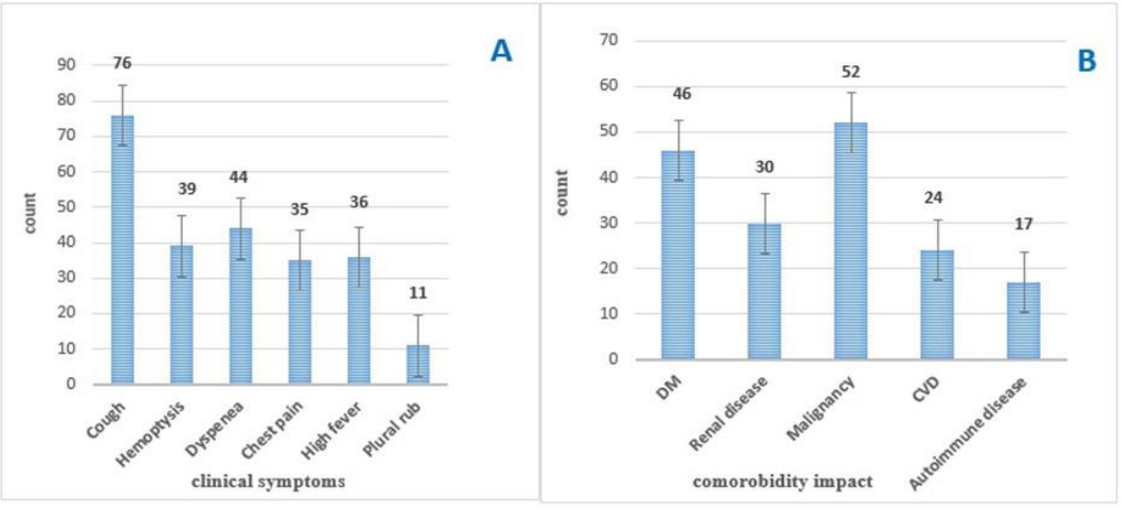

Fig. 2. Clinical symptoms and comorbidity impact hospitalized patients. (A) Frequency of clinical symptoms in hospitalized patients. (B) The recorded data of patients' files showed that malignancy, diabetes mellitus (DM), cardiovascular disease (CVD), renal disease, malignancy, and autoimmune disease had comorbidity impacts in hospitalized patients.

Table 2. Results of the direct examination and culture

Positivity rates of the methods	Direct examination (+) and culture (-)			Direct examination and culture (+)		
	Hyphae	Budding yeast cells	Total	Mold	Yeast	Total
Frequency of 360 patients	3	10	*13*	18	96	114
Percentage (%)	0.83	2.7	*3.6*	5	26.6	31.6

Fig. 3. Out of 12 *Aspergillus* isolates, 4 (33.3%) were isolated from patients with COPD, 3 (25%) from asthmatic patients, 1 (8.3%) from ILD patients, 2 (17%) from patients with bronchiectasis, and 2 (17%) from pulmonary TB patients. Out of 96 *candida* isolates, 41 (43%) were isolated from patients with COPD, 19 (19.7%) from asthmatic patients, 7 (7.3%) from ILD, 11 (11.4%) from patients with bronchiectasis, and 18 (18.6%) from pulmonary TB patients. Also, *Rhizopus* genus was isolated from patients with COPD, *Thrichosporon* from sthmatic patients, *Scedosporium* from ILD patients, *Geotrichum* from patients with bronchiectasis, and *Alternaria* from pulmonary TB patients. The correlation between isolated fungi and underlying lung diseases is represented in Table 3.

Yeast identification based on the RFLP-PCR method. Utilizing a PCR–RFLP method, we identified medically significant *Candida* species using the universal primers ITS1 and ITS4 to amplify the ITS1 and ITS2 region and 5.8S in the rDNA gene. In the current study, the size of PCR Amplicon for *C. albicans, C. tropicalis, C. krusei, C. parapsilosis,* and *C. glabrata* were 537, 526, 510, 530, and 881 bp, respectively. Also, the size of *MspI* -RFLP fragments for *C. albicans, C. tropicalis, C. krusei, C. parapsilosis,* and *C. glabrata* were 239-298, 186-340, 250-260, 530 and 320-561 bp, respectively. The results showed that all *Candida* spp. detected via microbiological methods were confirmed by the RFLP-PCR assay (Fig. 4).

Mold identification based on PCR and sequencing reference method. In this investigation, fragments of the *tubulin* gene (benA) were amplified and sequenced using Bt2a and Bt2b primers for the identification of *Aspergillus* spp. 11 of 12 (91.6%) *Aspergillus* spp. that were detected via microbiological methods were confirmed by using the primers Bt2a and Bt2b, and 1 (8.4%) isolate were negative. The negative results may be attributed to the isolated species not being genetically the same so that it can be detected by Bt2a and Bt2b, or a mutation had occurred with the isolated species and changed the nucleotide sequence which was not associated with the mentioned primer. Isolated *Aspergillus* strains produced a PCR product of the tubulin gene with sizes ranging from 550 to 600 bp. In

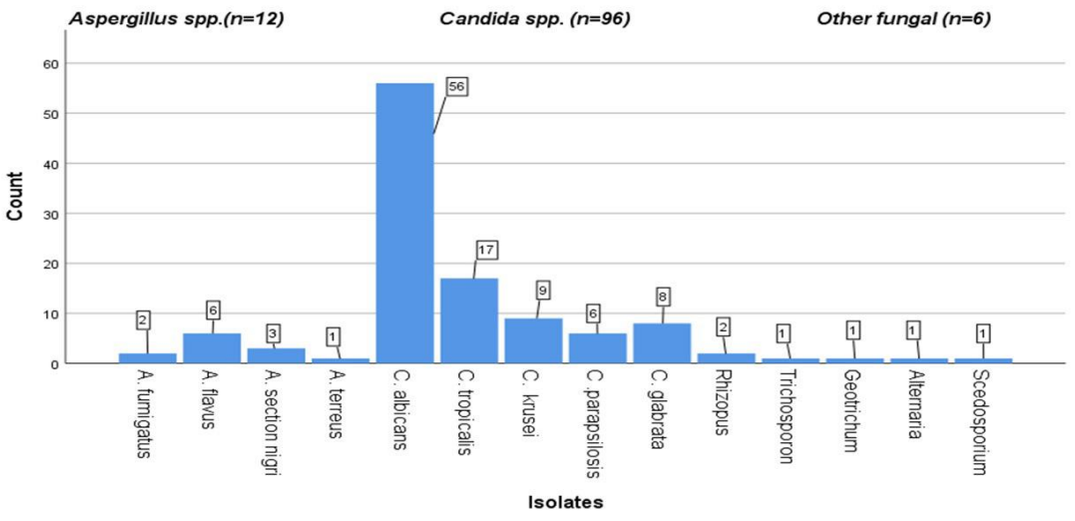

Fig. 3. Distribution of the fungal isolates concerning the diagnostic groups (n=114).

Table 3. Correlation between fungal isolates and underlying lung diseases.

Underlying lung diseases	COPD	Asthma	Bronchiectasis	ILD	TB	Other
Isolated *Aspergillus* spp. n=12 (10.5%)	4 (3.5%)	3 (2.5%)	1 (0.8%)	2 (1.7%)	2 (1.7%)	0
Isolated *Candida* spp. n=96 (84%)	41 (36%)	19 (16.7%)	7 (6.2%)	11 (9.3%)	18 (15.6%)	0
Isolated other fungal. n=6 (5.5%)	2 (1.5%)	2 (1.8%)	0	0	2 (1.7%)	0
Total=114 (100%)	47 (41%)	24 (21%)	8 (7%)	13 (11%)	22 (19%)	0

COPD, Chronic obstructive pulmonary disease; ILD, interstitial lung disease; TB, tuberculosis

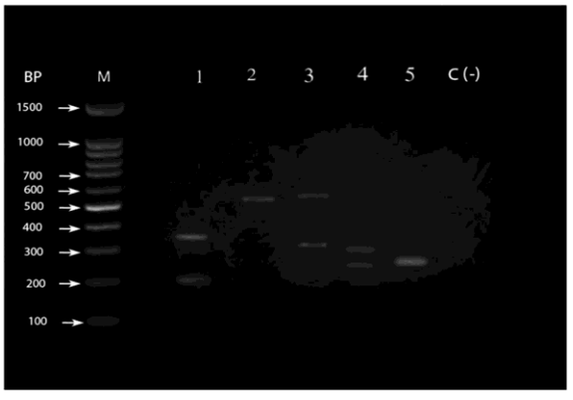

Fig. 4. Agarose gel electrophoresis of restriction digestion by the MspI enzyme of *Candida* strains in PCR- RFLP. lane 1, *C. tropicalis*; lane 2, *C. parapsilosis*; lane 3, *C. glabrata*; lane 4, *C. krusei*; lane 5, *C. albicanse*; M lane is 100 bp DNA markers, BP; Base pair, C lane is control negative.

this study, *Rhizopus*, *Thrichosporon*, *Scedosporium*, *Geotrichum* and *Alternaria* were identified according to mycological method.

Antifungal susceptibility testing (AFST). The ranges MIC/MEC, ECVs, and the distribution of MICs/MECs of four antifungal agents against the 40 *candida* isolates and 12 *Aspergillus* isolates are presented in Tables 4 and 5. Due to the pharmaceutical limitations of this project, drug sensitivity was conducted for 40 samples out of 96 *Candida* species and randomly selected for 12 /56 *C. albicans* samples and 11/17 *C. tropicalis* samples. In the present study, the susceptibility/resistance of the strains to each antifungal was evaluated according to the breakpoints and ECVs defined by CLSI and/or several researchers. The result of AFST for antifungal agents showed that

Table 5. Antifungal susceptibility pattern of antifungal agents against the *Aspergillus* isolates.

Isolates	No. of isolates with respective MICs/MECs (µg/ml)										MIC profile (µg/ml)		ECV profile			Susceptibility profile	
	Antifungal	0.031	0.062	0.125	0.25	0.5	1	2	4	8	>16	MIC range	GM	ECV	≤ECV	>ECV	WT	NWT
A. flavus (6)	AMB VRC	1	-	1	-	1	-	1	2	-	-	0.031-8	1.6	4	3	3	6	0
	ITC	2	1	-	1	2	-	-	-	-	-	0.031-0.5	0.34	1	3	3	6	0
	*CAS	1	2	-	-	3	-	-	-	-	-	0.031-0.5	0.53	1	6	0	6	0
	AMB	2	3	1	-	-	-	-	-	-	-	0.031-0.12	0.12	NA	NA	NA	6	0
A. fumigatus (2)	VRC	-	-	1	-	-	1	-	-	-	-	0.0125-1	0.56	2	2	0	2	0
	ITC	1	-	-	-	1	-	-	-	-	-	0.031-0.5	0.26	1	2	0	2	0
	*CAS	1	-	-	-	1	-	-	-	-	-	0.25-0.5	0.26	1	2	0	2	0
	AMB	1	1	-	-	-	-	-	-	-	-	0.031-0.62	0.04	NA	NA	NA	2	0
A. section nigri (3)	VRC	1	-	-	-	-	2	-	-	-	-	0.031-1	1.01	2	3	0	3	0
	ITC	-	-	-	1	2	-	-	-	-	-	0.25-0.5	0.62	2	3	0	3	0
	*CAS	-	-	-	-	1	2	-	-	-	-	0.5-1	1.25	2	3	0	3	0
	AMB	1	1	1	-	-	-	-	-	-	-	0.031-0.12	0.07	NA	NA	NA	3	0
A.terreus (1)	VRC	-	-	-	-	-	-	-	1	-	-	4	4	4	1	0	1	0
	ITC	-	-	-	-	-	1	-	-	-	-	1	1	1	1	0	1	0
	*CAS	-	-	-	-	1	-	-	-	-	-	0.5	0.5	1	1	0	1	0
	-	-	1	-	-	-	-	-	-	-	-	0.06	0.06	NA	NA	NA	1	0

MIC: Minimum inhibitory concentration; MEC: Minimum effective concentration; GM: Geometric Mean; AMB: Amphotericin B; ITC: itraconazole; VRC: voriconazole; CAS: caspofungin; WT: wild-type; ECV: epidemiologic cutoff values, NA: not applicable; *for caspofungin MEC Range determined. (µg/ml)

Table 4. Antifungal susceptibility pattern of antifungal agents against the *Candida* isolates

Isolates	No. of isolates with respective MICs (µg/ml)										MIC profile (µg/ml)				ECV profile			Susceptibility profile		
	Antifungal	0.031	0.062	0.125	0.25	0.5	1	2	4	8	>16	MIC range	MIC_{50}	MIC_{90}	GM	ECV	≤ECV	>ECV	S	SDD	R
C. albicans (12/56)	AMB	2	-	1	1	6	1	1		-	-	0.031-2	0.5	0.5	1.07	2	12	0	*	*	*
	VRC	7	-	1	2	-	-	1	-	-	1	0.031-16	0.031	0.25	3.7	0.06	7	5	8	2	2
	ITC	9	-	-	-	1	1	-	-	1	-	0.031-8	0.031	1	2.4	2	11	1	9	1	2
	CAS	8	1	1	2	-	-	-	-	-	-	0.031−0.25	0.031	0.25	0.23	0.25	12	0	12	0	0
C. krusei (9)	AMB	2	1	-	1	-	2	1	1	-	1	0.031-16	1	ND	3.4	2	7	2	*	*	*
	VRC	2	-	4	1	-	-	-	1	-	1	0.031-16	0.25	ND	4.1	0.5	7	2	7	0	2
	ITC	4	2	-	-	1	-	1		-	1	0.031-16	0.125	ND	3.7	2	7	2	7	0	2
	CAS	8	1	-	-	-	-	-	-	-	-	0.031-0.62	0.031	ND	0.15	0.5	9	0	9	0	0
C. tropicalis (11/17)	AMB	4	-	1	2	3	1	-	-	-	-	0.031-1	0.031	0.5	0.67	2	11	0	*	*	*
	VRC	5	-	1	3	1	-	-	-	-	1	0.031-16	0.031	0.25	3.5	0.12	5	6	6	4	1
	ITC	7	-	3	1	-	-	-	-	-	-	0.031-0.25	0.031	0.12	0.028	2	11	0	11	0	0
	CAS	8	2	1	-	-	-	-	-	-	-	0.031-0.12	0.031	0.062	0.16	0.5	11	0	11	0	0
C. glabrata (8)	AMB	3	-	1	2	-	1	1	-	-	-	0.031-2	0.031	ND	0.74	2	8	0	*	*	*
	VRC	5	2	-	1	-	-	-	-	-	-	0.031-0.25	0.031	ND	0.25	0.25	8	0	*	*	*
	ITC	5	-	1	-	2	-	-	-	-	-	0.031-0.5	0.031	ND	0.42	2	8	0	8	0	0
	CAS	5	2	-	1	-	-	-	-	-	-	0.031-0.25	0.031	ND	0.17	0.5	8	0	7	1	0

MIC: Minimum inhibitory concentration; GM: Geometric Mean; AMB: Amphotericin B; ITC: itraconazole; VRC: voriconazole; CAS: caspofungin; S: susceptible; SDD: susceptible-dependent-dose; R: resistant; ECV: epidemiologic cutoff values, ND: not determined; *Breakpoints not provided by CLSI document M27-S4.

(9/40, 22.5%) *Candida* isolates were resistant, and the highest rate of resistance was related to VRC agent (5/9, 55.5%). Resistance to antifungal agents was not observed among *Aspergillus* isolates which indicated that all isolates were within the WT. In this study, MIC_{50} and MIC_{90} were determined for numbers above 10 isolates. For each drug species pair, the MIC_{50} values differed in *candida* isolates, indicating that in all cases the MIC_{50} obtained by inspection reasonably reflected the central tendency of the antifungal susceptibility of the population.

DISCUSSION

The present study was conducted in Tabriz -Northwest Iran, focusing on patients admitted to different hospitals in Tabriz city with underlying lung disease. This study represents the first attempt to isolate fungal species, examine their molecular characteristics, and assess them in vitro susceptibility to four antifungal agents. Among 360 patient samples, 114 fungal isolates (31.6%) were identified. This prevalence was consistent with studies by Rafat et al. from Gilan, Iran, and Akhtar et al. from Bhagalpur, India, who reported similar findings (18, 19). However, a study by Ahmed et al. in Egypt showed a notably higher prevalence (66.6%), which may reflect the broader spectrum of underlying diseases in their study population (6). In our study, as in the findings of Rafat et al. (18), in positive cases, the male-to-female ratio was 63 to 51, and there was no significant difference in the prevalence of fungal elements isolated from symptomatic patients hospitalized in pulmonary units between the genders. Similar exposure to pollution sources due to equal socio demographic conditions, occupations, and societal roles may account for the observed similarity between the two gender groups. In our study, the predominant age group among hospitalized patients ranged from 11 to 49 years, yet the highest occurrence of fungal isolates was noted in individuals aged 45 to 75 years. The results of this study are similar to the findings of other studies from different countries (20, 21). This finding suggests that fungal colonization rates likely increase with age and the presence of underlying conditions, such as DM.

Direct microscopic examination, used to detect hyphal forms or budding yeast cells in clinical specimens, is a rapid and cost-effective diagnostic method (22). Our study showed that microscopic examination sensitivity was 11.4% in respiratory samples. This technique showed sensitivity only for 13 (11.4%) in respiratory samples, meaning it failed to detect 101 (88.6%) of the fungal isolates identified via culture. These findings align with studies by Moqled et al. (23) in Saudi Arabia, as well as Pagham et al. (24) from France, and and Agossou et al.(25) from France. In contrast, Njunda et al. (26) reported a much higher microscopic sensitivity of 90%. Additionally, Khodavaisy et al. showed negative microscopy results in patients with invasive pulmonary aspergillosis (27). While direct microscopic examination is less sensitive than culture, a negative result does not exclude fungal infection. Qualified mycological microscopes can enhance the sensitivity of microscopic examinations. The expertise of a mycologist is also crucial for accurate interpretation, for instance, in examining sputum samples, where hyphae are often visible alongside eosinophils and Charcot-Leyden crystals (22, 28).

In our study, *Candida albicans* was the most predominant fungal isolate in patients with underlying lung diseases, accounting for 49% (56 cases). This finding aligns with previous studies by Rafat et al. (18) and Spahr et al. (29) which also identified *C. albicans* as the leading cause of pulmonary fungal infections. Globally, *A. flavus* is regarded as the second most prevalent *Aspergillus* species in medical laboratories and hospitals, following *A. fumigatus.* However, unlike other regions, epidemiological studies in Iran have shown that *A. flavus* is the most common causative species of aspergillosis (30). The climatic and environmental conditions of the region and the availability of conditions for growth have been the most favoring factors in the abundance of *A. flavus* agents in Iran (31, 32). In the present study, 6 (50%) of the 12 clinical *Aspergillus* isolates were identified as *A. flavus*, consistent with findings from previous researches.

Several studies have examined the relationship between fungal infections and underlying lung diseases in hospitalized patients (33, 34). COPD, a prevalent inflammatory lung condition often linked to tobacco smoking, is one of the most significant risk factors. It is estimated that this disease can become the third cause of death in the future (33, 35). In our study, 47 (41%) COPD patients exhibited the highest rates of fungal colonization, with *Aspergillus* spp., *Candida* spp., and other fungal species isolated in

4 (3.5%), 41 (36%), and 2 (1.5%) cases, respectively. This observation is consistent with previous research, which identified COPD as a significant predisposing factor for colonization and infection by *Aspergillus* spp. (23, 33). Guinea et al. reported that 1.63% of COPD patients harbored *Aspergillus* spp. in their lower respiratory tract samples, with 22.1% showing probable IPA (36). Also, we observed that *Candida* colonization occurred in 41 patients (36%) with severe complications during the stable phase of COPD, which may be associated with worse clinical outcomes, particularly in critically ill patients. Delisle et al. (37) demonstrated an association between independent *C. albicans* colonization and prolonged hospital stays in ICU patients who met the clinical criteria for suspected ventilator-associated pneumonia (VAP). However, since *Candida* species can naturally be part of the respiratory system's normal flora, it is crucial for physicians to carefully evaluate clinical findings to confirm whether the *Candida* isolate is indeed associated with a lung infection.

Another objective of this research was to investigate the drug sensitivity of the isolates to AMB, ITC, VRC, and CAS. Treatment of fungal infections with an antifungal agent depends on the patient's clinical condition, and these drugs can also be used as prophylaxis in high-risk patients (33). Based on the CLSI interpretive criteria for the most predominant yeast isolates, (31/40, 77.5%) of *Candida* spp. were susceptible to AMB, ITC, VRC, and CAS, while resistance was observed in 9 out of 40 isolates (25.5%), predominantly to VRC (55.5%) (38-40). *Aspergillus flavus* isolates (50%) exhibited MICs $\leq$ 4 µg/ml to AMB, and besides this, all *Aspergillus* isolates were WT or susceptible to AMB, ITC, VRC, and CAS, providing promising therapeutic options for treating mold infections. Triazole resistance, particularly in *A. fumigatus*, is a growing concern in clinical settings (41). Unlike some studies that have reported triazole-resistant *Aspergillus* isolates, our findings showed no such resistance (42). A key aspect in performing antifungal susceptibility tests is the clinical relevance of *in vitro* MICs. The correlation of increased MICs with molecular resistance to triazole antifungals and treatment failure has been recognized, specifically for *Aspergillus* spp. (2). ITC remains a preferred treatment for chronic pulmonary aspergillosis, while VRC is first-line for invasive aspergillosis (IA). Recently introduced triazoles like isavuconazole and posaconazole offer additional treatment options for IA and prophylaxis in high-risk patients, such as acute myeloid leukemia (43), but their availability in resource-limited settings remains challenging.

We utilized a PCR-RFLP method to identify medically significant *Candida* species. Our findings were consistent with those of other published studies (44, 45), which also reported similar results when employing CHROMagar and PCR-RFLP methods to detect various *Candida* species. However, Shokohi et al.(12) and Hassanpour et al. (8) found that CHROMagar could be unreliable due to color interpretation issues, suggesting the need for standardized *Candida* strains to minimize diagnostic errors. Additionally, the expertise of a mycologist during fungal identification is crucial.

Lastly, we applied PCR and sequencing reference methods to identify *Aspergillus* spp. In this method, the identification marker for *Aspergillus* spp. was the *beta-tubulin* gene. In our study, amplification with the *beta-tubulin* gene revealed that all isolates of *Aspergillus* spp. were positive and had amplicon sizes of 550-600 bp. These results were similar to those obtained in previous studies (11, 46, 47). In other research, *beta-tubulin* gene amplicon sizes ranged from 432 to 550 bp for *Aspergillus*, *Penicillium*, and other fungal species (48). While the ITS region serves as a universal primer for fungi, its utility in identifying most fungi to the species level is limited due to their low variability and slow evolution. Reports have shown that *beta-tubulin* genes exhibit more variability than the ITS region. Therefore, it has been suggested to utilize alternative molecular markers, such as calmodulin (CMD) and RNA Polymerase II Second Largest Subunit (RPB2), to enhance the accurate identification of fungal species and facilitate phylogenetic analysis.

CONCLUSION

The most important finding of this study was the isolation of 114 fungal isolates from 360 patients through direct examination and culture. Diabetes, transplantation, and malignancies were paramount risk factors in hospitalized patients with underlying pulmonary disease. *C. albicans* and *A. flavus* were the most common yeast and mold species responsible for pulmonary fungal colonization and/or infection among patients. In addition, antifungal VRC was more active than ITC, especially against *A. flavus.*

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