International Journal of Molecular and Clinical Microbiology 1 (2014) 358-364

International Journal of
Molecular and Clinical Microbiology

Identification and Antifungal Susceptibility Testing of *Candida* species isolated from Bronchoalveolar Lavage samples

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ARTICLE INFO

Article history:
Received 13 February 2014
Accepted 17 May 2014
Available online 1 June 2014

Keywords:
Antifungal Susceptibility Testing, Candida, Bronchoalveolar Lavage samples

ABSTRACT

The frequency of fungal infections in immunocompromised patients, particularly by *Candida* species, has increased in recent years. Colonization by *Candida* species in respiratory tract in susceptible hosts may play an important role to precede disseminated candidiasis. This study was designed to identify *Candida* species from bronchoalveolar lavage (BAL) samples and determination of antifungal susceptibility of isolates to Ketoconazole, Clotrimazole, Fluconazole and Nystatin by disk diffusion method. Sampling was conducted between from 2011 to 2014 years. Three hundred and eighty four patients who were suspected to invasive fungal infections were enrolled in the study. Clinical specimens were studied for direct microscopic examination and culture. The antifungal activity test for *Candida* species isolated from BAL samples was performed by using disk diffusion, according to *CLSI* documents M44-A2. Eighty seven (%22.66) patients showed the symptoms, signs and predisposing factors for pulmonary fungal infections. The isolated species were identified as follows: *C.albicans*, 31 (67.39%); C.*glabrata*, 9 (19.56 %)*; C.krusei*, 3 (6.5%); *C.parapsilosis*, 2 (4.3%); and *C.tropicalis* , 1 (2.25%). In this study, resistance to antifungal agents were seen to Ketoconazole, 2 (4.38%), Clotrimazole 1 (2.17%) and Fluconazole, 4 (8.69%). Determination of antifungal sensitivity of the isolated yeast species should be the basis of rational and successful therapy.

1. Introduction

Fungal diseases are as an important source of nosocomial infections, particularly among immunocompromised individuals (Richardson et al., 2005). Mortality rate of invasive Aspergillosis ranges from 30% to 70%, but this rate for Candidemia in immunosuppressed patients is 40% (Knox et al., 2009). Colonization of the tracheobronchial tree seems to be quite

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common, while invasive pulmonary infection by *Candida* species is rare in immunocompetent patients in contrast to immunosuppressed patients (Knox et al., 2009). Several studies indicated that the frequency of *Candida* pneumonia between 0.23 to 8%; with the highest incidence of candidiasis in immunocompromised patients (El-Ebiary et al., 1997; Haron et al., 1993). In recent decades, the use of antibiotics, corticosteroids, immunosuppressive therapy, catheters, prosthetic devices, the emergence of AIDS and organ transplantation, have created clinical challenges with new deep mycoses (Abu-Elteen et al., 2012). Lung is one of the main organs involved in disseminated candidiasis. The autopsies from patients with disseminated candidiasis have shown the pulmonary involvement in the half of these patients. Clinical symptoms of pulmonary candidiasis are non-specific with considerable mortality (Knox et al., 2009). Therefore, early diagnosis and treatment of disease is critical. Bronchoscopy with bronchoalveolar lavage (BAL) is a significant way for the diagnosis of pulmonary infections and cancer (Meduri et al., 1991). Flexible fiberoptic bronchoscopy is moderately safe and minimally invasive means for taking bronchoalveolar lavage (BAL) fluid (Rajesh Kumar and Jain, 2014). However the proven candidiasis is made by histopathology, but lung biopsy in many cases are not feasible (Huang et al., 2006). Although isolation of *Candida* species from the BAL in patients with pneumonia does not mean *Candida* infection but it can assist in early detection of susceptible individuals (El-Ebiary et al., 1997). Colonization by *Candida* species in respiratory tract in susceptible hosts may play an important role to precede disseminated candidiasis. On the other hand, the observation of fungal colonization in more than two areas of the body and blood cultures, pleural fluid, or other sterile fluids without biopsy may be helpful to make a decision to initiate treatment (Guarner et al., 2011). The aim of this study was to evaluate the identification of *Candida* species isolated from BAL samples from patients who were susceptible to disseminated candidiasis and antifungal susceptibility testing of them to Ketoconazole, Clotrimazole, Fluconazole and Nystatin by disk diffusion method.

2. Materials and Methods

2.1. Patients

Sampling was conducted between in 2011-2014. Three hundred and eighty four patients who were suspected to invasive fungal infection from Shariati Hospital Tehran, Iran were enrolled in the study. The inclusion criteria for patients were to have at least one of the following conditions: blood cancer patients, persons with cancer and those who have at least 3 months before admission chemotherapy, recipients of bone marrow, lung, liver, kidney; use of medications corticosteroid; recipients of immunosuppressive medications including cyclosporine, tacrolimus, methotrexate, cyclosphosphamid, People with HIV, severe neutropenia, COPD, cirrhosis of the liver, GVHD, indwelling central IV, bacterial infection, mucosal colonization. In addition to host factors, clinical symptoms and laboratory diagnostic methods were considered for approval.

2.2. Laboratory work

Fiberoptic bronchoscopy (Olympus BF20D) with BAL was performed thereafter if feasible. The sampling area was selected based on the infiltrate location on the chest radiograph. Then, 50 ml sterile normal saline was injected through the device. The suction channel of the bronchoscope was used to instill and aspirate 25-30 ml fluid. Afterwards, clinical samples were collected in sterile tubes and immediately were transferred to Laboratory Medical Mycology at Mazandaran University of Medical Sciences.

2.3. Direct microscopic examination (DME) and culture BAL

BAL samples were diluted with an equal volume of 0.5% Pancreatin and incubated for 1-2 h at 25° C with shaking. Afterward they were centrifuged for 5 min at 5000 rpm, the supernatant discarded. Remaining sediment was used for direct smear using CalcuFlour white

staining and culture. Sabouraud Dextrose Agar containing with Chloramphenicol (Sc) was used for the isolation of *Candida* species. The inoculated media were incubated at 30°C for 5 days. In each clinical sample, yeasts with different morphological characteristics were isolated and stored at -20°C for further identification. *Candida* species isolated from different of BAL samples were confirmed by phenotypic (Corn Meal Agar, Chlamydospore formation, CHROMagar and Germ tube test) and genotypic PCR-RFLP approaches.

2.4. Antifungal susceptibility testing

The antifungal activity test was performed by using disk diffusion, according to *CLSI* documents M44-A2. A 0.5 McFarland suspension of each isolate was swabbed in three directions on Mueller-Hinton agar supplemented with 2% glucose and methylene blue (0.5µg/ml). These inoculated plates were left to dry for at least 20 min, after which blank paper disks (6.3-mm diameter; Padtan-Teb, Iran) Containing Ketoconazole (10 µg/µl), Clotrimazole (50 µg/µl), Fluconazole (100 µg/µl), and Nystatin (100 U) disks were prepared and used for determination of susceptibility (Arendrup et al., 2011). The standard isolates of *C.albicans* (ATCC 76615, as resistant strain, and ATCC 10231, as susceptible strain) were also used for quality control of each test.

3. Results

3.1. Patient's data and candida species isolated

Out of 384 admitted patients, 167 (%43.50) were female. The age range of patients was between 14-85 years old. Of 384 patients referred to Bronchoscopy, 87 (%22.66) showed symptoms, signs and predisposing factors for pulmonary fungal infections (Autoimmune, Neutropenia, GVHD, Lung Transplantation, Malignancies, Tuberculosis, Renal Dialysis, Pneumonia, Leukemia, Corticosteroids Therapy, Usage immunosuppressive, Chemical Warfare, Diabetes, Renal Transplantation, Hodgkin lymphoma, bacterial and viral infection, etc…). Of these 87 patients with underlying predisposing factor 39 (%44.83) were female. Fever, cough, sputum and dyspnea were the most common presented symptoms, but pleuritic chest pain and hemoptysis are found in approximately a quarter of patients. Out of 87 patients, 31 (%35.63) cases showed budding yeast cells and Pseudohyphae in DME. Forty six (%52.87) BAL samples were positive for *Candida* spp. growth on CHROM agar. The isolated *Candida* species were identified as follows: *C.albicans*, 31 (67.39%); *C.glabrata*, 9 (19.56 %); *C.krusei*, 3 (6.5%); *C.parapsilosis*, 2 (4.3%); and *C.tropicalis*, 1 (2.25%). The most underlying conditions were diabetes mellitus (13; 15.3%), renal failure (9; 10.3%) and renal transplantation. Table 1 show the number and species of *Candida* which isolated from studied patients based on underlying diseases, and in-vitro antifungal susceptibility patterns of *Candida* species.

3.2. Antifungal Susceptibility Patterns

C.albicans, the most isolated species, was sensitive to Ketoconazole, Clotrimazole, Fluconazole and Nystatin in 58.7%, 63.13%, 56.51%, and 67.39% cases, respectively. Of the 9 isolates of *C.glabrata*, 6 (13.04%) were sensitive to Ketoconazole, 7 (15.23%) to Clotrimazole, 5 (10.98%) to Fluconazole and 7 (15.23%) to Nystatin. Among the 3 isolates of *C.krusei*, 2 (4.34%) were found to be sensitive to Ketoconazole, Clotrimazole, Fluconazole, but (85%) all of them were sensitive to Nystatin. 2 isolates of *C.parapsilosis*, 2 (4.3%) and *C.tropicalis* 1 (2.25%), were sensitive to Ketoconazole, Clotrimazole, Fluconazole and Nystatin. Antifungal susceptibility testing revealed that none of the isolates tested were resistant to Nystatin, but two isolates (4.38%) resistant to Ketoconazole, one isolate (2.17%) resistant to Clotrimazole and four isolates (8.69%) resistant to Fluconazole. The dose dependent susceptible of *Candida* species were seen as follows: Ketoconazole 6 (13.05%), Clotrimazole 4 (8.69%), Fluconazole 6 (13.05%), and Nystatin, 2 (4.38%) (Table 3).

Table 1. Predisposing factors of candidiasis, Clinical isolated of *Candida* species and in-vitro antifungal susceptibility patterns of *Candida* species to, Ketoconazole, Clotrimazole, Fluconazole, and Nystatin

Predisposing factors	Number	Percent (%)	Organisms	Number	Ket			Clo			Flu			Nys		
					S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R
Diabetes Mellitus	13	15.3	*C. albicans*	4	4	-	-	4	-	-	4	-		4	-	-
			C. glabrata	2	2	-	-	2	-	-	2	-	-	2	-	-
			C. krusei	1	1	-	-	1	-	-	1	-	-	1	-	-
			C. parapsilosis	1	1	-	-	1	-	-	1	-	-	1	-	-
			C. tropicalis	1	1	-	-	1	-	-	1	-	-	1	-	-
AML or blastic phase CML	5	6	*C. albicans*	2	2	-	-	2	-	-	2	-	-	2	-	-
			C. glabrata	1	-	1	-	1	-	-	-	1	-	1	-	-
ALL	4	4.5	*C. albicans*	2	2	-	-	2	-	-	2	-	-	2	-	-
CML	6	6.8	*C. albicans*	3	3	-	-	3	-	-	3	-	-	3	-	-
			C. glabrata	1	-	-	1	-	1	-	-	-	1	-	1	-
Aplastic anaemia or myelodysplastic sydrome	2	2.2	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
Lymphoma	5	6	*C. albicans*	3	3	-	-	3	-	-	3	-	-	3	-	-
CLL or myeloma	6	6.8	*C. albicans*	3	3	-	-	3	-	-	3	-	-	3	-	-
			C. glabrata	2	2	-	-	2	-	-	1	-	1	2	-	-
ITP	1	1.1	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
Renal Failure and Renal Transplantation	9	10.3	*C. albicans*	5	4	1	-	5	-	-	2	2	1	5	-	-
			C. glabrata	1	1	-	-	1	-	-	1	-	-	1	-	-
Infectious Diseases (Tuberculosis)	8	9.1	*C. albicans*	3	2	-	-	3	-	-	3	-	-	3	-	-
			C. krusei	1	1	-	-	1	-	-	1	-	-	1	-	-
Autoimmune Disease	2	2.2	*C. albicans*	1	1	-	-	1	-	-	1	-	-	1	-	-
Immunosuppressive Therapy	7	8	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
			C. parapsilosis	1	1	-	-	1	-	-	1	-	-	1	-	-
Bone Marrow Malignancy	3	3.5	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
			C. krusei	1	-	1	-	-	1	-	-	-	1	1	-	-
Chronic Granulomatous Disease(CGD)	6	6.8	*C. albicans*	3	1	2	-	1	1	1	1	2	-	3	-	-
			C. glabrata	2	1	1	-	1	1	-	1	1	-	1	1	-
Pancytopenia	3	3.5	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
Anaemia (Hb < 9%)	1	1.1	*C. albicans*	0	-	-	-	-	-	-	-	-	-	-	-	-
Usage of antibiotics with broad spectrum	6	6.8	*C. albicans*	2	2	-	-	2	-	-	2	-	-	2	-	-
Total	87	100		46	39	6	1	41	4	1	36	6	4	44	2	0

AML: acute myeloid leukaemia; ALL: acute lymphoid leukaemia; CML: chronic myeloid leukaemia; CLL: chronic lymphocytic leukaemia: ITP: idiopathic thrombocytopenic purpura; BMT: bone marrow transplantation; Ket: Ketoconazole; Clo: Clotrimazole; Flu: Fluconazole; Nys: Nystatin; S: Susceptible; SDD: Susceptible Dose Dependent; R:Resistance.

Table 2. Growth inhibition zones interpretation for antifungal drugs

Antifungal drugs	Zone diameter in mm		
	Sensitive	Dos dependent	Resistance
Ketoconazole	≥30	29-23	≤22
Clotrimazole	≥20	19-12	≤11
Fluconazole	≥19	15-18	≤14
Nystatin	≥25	17-24	<16

Table 3. Susceptibility and percentages of isolates of *Candida* species to Antifungal drugs

Antifungal drugs	*Susceptibility*	*C. albicans*	*C. glabrata*	*C. krusei*	*C.parapsilosis*	*C.tropicalis*	Total
	Sensitive	27 (58.7%)	6 (13.04%)	2 (4.34%)	2 (4.3%)	1 (2.25%)	38(82.63%)
Ketoconazole	Susceptible Dose Dependent	3 (6.56%)	2 (4.29%)	1 (2.21%)	0 (0.0%)	0 (0.0%)	6 (13.05%)
	Resistance	1 (2.13%)	1 (2.18%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (4.38%)
	Total	31 (67.39%)	9 (19.56 %)	3 (6.5%)	2 (4.3%)	1 (2.25%)	46(100%)
Clotrimazole	Sensitive	29 (63.13%)	7 (15.23%)	2 (4.34%)	2 (4.3%)	1 (2.25%)	41(89.14%)
	Susceptible Dose Dependent	1 (2.13%)	2 (4.33%)	1 (2.21%)	0 (0.0%)	0 (0.0%)	4 (8.69%)
	Resistance	1 (2.13%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.17%)
	Total	31 (67.39%)	9 (19.56 %)	3 (6.5%)	2 (4.3%)	1 (2.25%)	46(100%)
Fluconazole	Sensitive	26 (56.51%)	5(10.9%)	2 (4.34%)	2 (4.3%)	1 (2.25%)	36(78.26%)
	Susceptible Dose Dependent	4 (8.75%)	2 (4.33%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	6(13.05%)
	Resistance	1 (2.13%)	2 (4.33%)	1 (2.21%)	0 (0.0%)	0 (0.0%)	4 (8.69%)
	Total	31 (67.39%)	9 (19.56 %)	3 (6.5%)	2 (4.3%)	1 (2.25%)	46(100%)
Nystatin	Sensitive	31(67.39%)	7 (15.23%)	3 (6.5%)	2 (4.3%)	1 (2.25%)	44(95.62%)
	Susceptible Dose Dependent	0 (0.0%)	2 (4.33%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (4.38%)
	Resistance	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Total	31 (67.39%)	9 (19.56 %)	3 (6.5%)	2 (4.3%)	1 (2.25%)	46(100%)

4. Discussion

The fungal infection of the respiratory tract is the major cause of morbidity and mortality in immunocompromised patients. This issue is significance in patients treating with cytotoxic drugs, using corticosteroids, patients undergoing bone marrow, lung, and renal transplantation, and patients with acquired immunodeficiency, severe neutropenia, COPD, cirrhosis of the liver and GVHD (Richardson et al., 2005). Isolation of *Candida* from sputum, tracheal aspirates, BAL, and even lung tissue may simply represent colonization rather than infection (Knox et al., 2009). BAL represents an additional tool in the assessment of the health status of the lung for mycologists that can facilitate the diagnosis of various diffuse fungal lung diseases. As the BAL samples are competent to provide cells and solutes from the lower respiratory tract in the present study we use the BAL samples from the patients suspected to IFI in order to evaluation of *Candida* colonization in respiratory tract and antifungal susceptibility of isolated *Candida* species against some common antifungal drugs. In this present study, *C.albicans* (67.39%) was the most predominant species isolated from BAL samples. *C.tropicalis* had the least frequency. Our data had some similarity with the data obtained from other previous studies from different countries. Delisle et al. performed a study to investigate *Candida* colonization and its associated risk factors, and to examine the clinical outcomes in patients with clinical suspicion of ventilator-associated pneumonia with concordant *Candida* colonization (n = 114) and without *Candida* colonization (n = 525) (Delisle et al., 2008). Of the 639 eligible patients, 114 (17.8%) were colonized with *Candida* in the enrollment culture. We found *Candida* colonization confined to respiratory tract secretions in 31 (35.63%) of 87 patients. Delisle et al. was accounted *C.albicans* for in 65.3% of samples, whereas other non-albicans sp were found in 6.7% of airway specimen. In our study *C.albicans* were found 31 (67.39%) and non-albicans sp 15 (32.61%). In El-Ebiary et al study in 1997 on BAL samples, *C.albicans* (80%), and *C.krusei* (10%), were isolated, respectively. In another study at Taiwan (2001) in BAL samples of patients studied, the frequency of *C.albicans* was the first rank followed by *C.glabrata* and *C.tropicalis* (Rano et al., 2001). In a study at the University of Texas in 2002 on BAL and Sputum specimens, *C.albicans* and *C.glabrata* were the most abundant species (Kontoyiannis et al., 2002). The majority of clinical symptoms in present study were low fever, cough, hemoptysis, weight loss, hypoxia, and dyspnea. We observed that 52.87% of patients recruited in our study were culture-positive for *Candida* species and demonstrated the presence of 35.63% budding yeast cells at DME. In our study the most common underlying condition were diabetes mellitus 15.3%, renal transplantation 10.3% and tuberculosis 9.1% that *Candida* species were isolated from 15.56%, 13.04% and 8.69% of the

samples, respectively. According to the emergence of resistant species among *Candida* species such as *C.glabrata* (35%) and *C.krusei* (75%), due to the use of azoles as prophylaxis for fungal infections, identification of *Candida* species is very important for proper treatment of patients. The increased hospital-acquired infections and drug resistance rate due to *C.albicans* and non-albicans species have been reported with high mortality rates (Krcmery et al., 2002; Wingard et al., 1991). In the present study, resistance to antifungal agents was seen for Ketoconazole (2; 4.38%), Clotrimazole (1; 2.17%) and Fluconazole, (4; 8.69%). There was no resistance to the drug nystatin. Antifungal susceptibility testing of our isolates revealed that all isolates was sensitive to nystatin. However, significant resistance (8.69%) was observed for fluconazole. Colombo et al., (2002) used a formerly described disk diffusion method to estimate the susceptibility profile of clinical *Candida* spp. isolates against fluconazole. A total of 50 yeast isolated from BAL samples were tested to, including the following species: *C.albicans* (48), *C.tropicalis* (1), and *Candida* spp. (1) and other yeasts (5). The majority (94%) of all tested yeast isolates were susceptible to fluconazole (Colombo et al., 2002). Furlaneto et al (2011) identifed *Candida* isolates obtained from blood 40 (19.2%), urine 111 (53.4%), tracheal secretion 37 (17.8%), and 20 nail/skin (9.6%) lesions from cases attended at the Hospital University de Londrina over a 3-year period and susceptibilities isolates was evaluating to fluconazole by EUCAST-AFST reference procedure. Out of of 37 *Candida* isolates including *C.albicans* (n=12), *C.tropicalis (n=8)* and *C.parapsilosis (n=17)*, most of the isolates were susceptible to fluconazole (Furlaneto et al., 2011). Biernasiuk et al who analyzed the drug susceptibility of *Candida albicans* isolated from two groups of chronic hepatitis C (group I, without antiviral therapy and group II, treated with peginterferon and ribavirin) against Amphotericin B, Flucytosine, Fluconazole, Itraconazole, Ketoconazole and Miconazole, 100% of *C.albicans* isolates were sensitive to Flucytosine and Amphotericin B. The resistant isolates resistant to Ketoconazole (6.67%) or Itraconazole (10%) were found in group I, while resistant to Miconazole (9.68%), Ketoconazole (19.35%), Itraconazole (22.58%) or fluconazole (3.22%) in group II (Biernasiuk et al., 2013). To our knowledge, no previous study has demonstrated a relationship between respiratory tract *Candida* infection and increased mortality. The findings of this study suggest that patients with *Candida* infection of respiratory tract secretions are at risk of worse outcome with possibly numerous contributing factors. Further prospective trials are required to confirm which patient characteristics are risk factors for respiratory tract secretions *Candida* infection and whether such colonization is independently associated with worse clinical outcomes. Due to increased fungal infections and drug resistance, therapeutic control of *Candida* spp. is very important. For the successful treatment of patients, assessment of drug sensitivity is required.

Acknowledgment

The participating patients are thanked for their kind cooperation, which was essential for the completion of the study. This study was supported by Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari (Iran).

Refereces

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