In Vitro Antifungal Susceptibility Profiles of *Candida albicans* Complex Isolated from Patients with Respiratory Infections

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Abstract- *Candidiasis*, the main opportunistic fungal infection has been increased over the past decades. This study aimed to characterize *C.albicans* species complex (*C.albicans*, *C.dubliniensis*, and *C.africana*) isolated from patients with respiratory infections by molecular tools and *in vitro* antifungal susceptibilities by using broth microdilution method according to CLSI M27-A3 guidelines. Totally, 121 respiratory samples were collected from patients with respiratory infections. Of these, 83 strains were germ tube positive and green colonies on chromogenic media, so initially identified as *C.albicans* species complex and subsequently were classified as *C.albicans* (89.15%), *C.dubliniensis* (9.63%), and *C.africana* (1.2%) based on PCR-RFLP and amplification of hwp1 gene. Minimum inhibitory concentration (MICs) results showed that all tested isolates of *C.albicans* complex were highly susceptible to triazole drugs. However, caspofungin had highest activity against *C.albicans*, *C.dubliniensis*, and *C.africana*. Our findings indicated the variety of antifungal resistance of *Candida* strains in different areas. These results may increase the knowledge about the local distribution of the mentioned strains as well as their antifungal susceptibility pattern which play an important role in appropriate therapy.

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Keywords: *In vitro* susceptibility; *C.albicans* complex; Caspofungin

Introduction

Candidiasis is the main opportunistic fungal infection which has been increased over the past decades (1). Invasive infection caused by *Candida* species are associated with high mortality ranging from 40% to 60% (2). Disseminated *Candidiasis* mainly involves the lungs. The autopsies from patients with disseminated *Candidiasis* have shown the pulmonary involvement in half of these patients which has considerable mortality (3).

C.albicans is a commensal and a constituent in the mucosa of healthy individuals. It's also the most common opportunistic human fungal pathogen. In the majority of epidemiological studies has been found to be the most common cause of superficial and systemic fatal infections in immunocompromised or critically ill patients. It is recognized as the fourth leading cause of nosocomial infections (4,5). Moreover, *C. dubliniensis* is closely related to *C.albicans* and shares many phenotypic properties traits with *C.albicans* such as the ability to form chlamydospores and germ tubes. However, it is often quite difficult to discriminate between the two species in clinical samples (5,6). Differences between these two species were most pronounced at the genetic variation and seemed to be important in medical mycology, *C. dubliniensis* exhibits increased adherence to epithelial cells and may have a higher propensity to develop azole antifungal drug resistance rather than *C.albicans* (7). Atypical *C.albicans* strains, i.e., *C. africana* have been reported in vaginitis, from African, German, Spanish, and Italian patients who considered an atypical chlamydospores-negative *C.albicans* strain (8). Based on molecular

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studies *C.africana* cannot be yet considered as a new species of *Candida*. These data suggest that *C.albicans* var. *africana* is a more suitable name for these atypical *C.albicans* strains (9). Epidemiology, virulence, and antifungal and intrinsic susceptibility pattern often vary among strains. Therefore a rapid and accurate identification of the species causing disease is crucial for primary antifungal regimens that be adjusted to the local epidemiological studies.

In the present study, we used a PCR-based molecular method for discriminating between *C.albicans*, *C.africana*, and *C. dubliniensis* by performing a single pair of primers targeting the *hwp1* gene. For this aim, we used *C.albicans* complex strains which isolated from patients with respiratory infection symptoms and identified by phenotypic and molecular methods. Antifungal susceptibility testing was conducted according to clinical and laboratory standard institute (CLSI) document M27-A3.

Materials and Methods

This study was performed in the Division of Molecular Biology, Department of Medical Mycology and Parasitology, Tehran University of Medical Sciences. Totally, 121 respiratory samples including sputum and broncho alveolar lavage (BAL) samples obtained from patients with respiratory infection symptoms. Among these samples, 83 strains were identified as C.albicans complex based on phenotypic methods (colony color on Candida Chrome agar medium (CHOROM agar Company, Paris, France) and germ-tube formation in serum at 37°C). Subsequently, confirmations were performed based on PCR-RFLP method. Briefly, genomic DNA was extracted using glass beads and the phenol/chloroform method and stored at -20°C prior to use (3,10). The contiguous ITS1-5.8S rDNA-ITS2 region was amplified by using PCR mixture containing 5 µl of 10x reaction buffer, 1.5 mM MgCl2, 0.4 mM dNTPs, 0.5 U of DNA Taq polymerase, 10 pmol of each ITS1 (5 ′ -TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′ -TCC TCC GCT TAT TGA TAT GC-3′) primers, and 2 µl of extracted DNA in a final volume of 25 µl. The amplification parameters consist of 35 cycles. The PCR condition was an initial denaturation at 94°C for 5 min, second denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min and 15 s, with a final extension step at 72°C for 8 min. The products resulting were visualized by ethidium bromide in 1% agarose gel electrophoresis. The restriction fragment was then obtained by digestion of 5µl PCR products with 0.5 µl of restriction enzyme *Msp I* (Fermentas, Vilnius, Lithuania) at 1.5 µl Tango buffer and 8 µl Molecular grade water in 37ºC for 2 h. Restriction fragments were separated on a 2% agarose gel in TAE buffer for 1.45 h at 100 V. The size of DNA fragments determined directly with a comparison of molecular size marker and distinct banding patterns which demonstrated in previous studies. The size of banding pattern for ITS PCR product (537 bp) and its fragments digested by *Msp I* (239 and 298 bp) was mentioned previously in *C.albicans* complex (10). The validated *C.albicans* strains were then used for further study concerning differentiation from *C. dubliniensis* as well as *C.africana*. Then, definite identification of *C.albicans*, *C.dubliniensis* and *C. africana* were performed by PCR amplification of the hyphal wall protein1 (*hwp1*) gene by using the forward 5′GCTACCACTTCAGAATCATCATC-3′ and reverse 5′ GCACCTTCAGTCGTAGAGACG-3′ primer pairs. Briefly, PCR reaction conditions were as follows: denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 58 °C for 40 s, and extension at 72 °C for 55 s, followed by a final extension at 72 °C for 10 min. PCR products were separated on a 1% (wt/vol) agarose gel. *C.albicans* isolates, including the reference showed the expected DNA fragment (~1000 bp), *C.dubliniensis* strains produced a fragment of (569 bp) and *C.africana* (~700 bp) (8).

In vitro antifungal susceptibility testing was performed for 83 identified strains by broth microdilution method according to the clinical and laboratory standard institute (CLSI) document M27-A3. MICs (minimum inhibitory concentrations) of four antifungal drugs; fluconazole (Pfizer Central Research, Sandwich, United Kingdom), itraconazole (Janssen Research Foundation, Beerse, Belgium), caspofungin (CAS, MerckSharp & Dohme, Haarlem, The Netherlands), and amphotericin B (AMB, Bristol-Myers Squib, Woerden, The Netherlands) were evaluated. Stock solutions of the drugs were prepared in the appropriate solvent according to the CLSI. The targeted final concentrations were for fluconazole (0.063-64 µg/ml), for caspofungin (008-8 µg/ml) and for amphotericin B and itraconazole (016-16 µg/ml). Aliquot in 96 well microdilution plates and store at -70°C until used. Final inoculum suspensions were prepared from 24 hr of *C.albicans* complex cultures ranged from 0.5×10^3 to 2.5×10^3 CFU/ml by spectrophotometr. The plates were then incubated at 35°C for 48 hr. Visual readings were performed with the

help of a mirror. The MICs endpoints of fluconazole have been defined as the level which induced a prominent reduction of growth (50% inhibition compared to drug-free growth control). Visible fungal growth which can be inhibited 100% is considered as MIC for amphotericin B, itraconazole and caspofungin. *In vitro* susceptibilities were then evaluated according to the currently established interpretation criteria of the CLSI. For fluconazole, strains are considered as susceptible when the MIC≤8 µg/ml, susceptible dose dependent MIC=16-32 µg/ml, and resistant with MIC≥64 µg/ml. Corresponding criteria for itraconazole MIC≤0.125 µg/ml is susceptible, MIC=0.25-0.5 µg/ml dose dependent and MIC≥1 µg/ml is resistant. Amphotericin B considered as resistant with ≥2 µg/ml and susceptible in <1µg/ml MICs. For caspofungin, the fungal growth is considered as susceptible and resistant when MIC <2 µg/ ml and MIC >2µg/ ml, respectively (11-13). *Candida* parapsilosis (ATCC 22019) was chosen as quality controls to be used with every new series of MICs plates.

Results

From all tested isolates that initially identified as *C.albicans* complex, 89.15%, 9.63%, 1.2% was identified as *C.albicans*, *C. dubliniensis*, and *C. africana* respectively, by amplification of the *hwp1* gene (Figure 1). *Candida albicans* was the dominant *Candida* species (n=74; 89.15 %) obtained from respiratory samples. The comparative *in vitro* susceptibilities of the *C.albicans* complex isolated from patients with respiratory infection symptoms against antifungal agents summarized in Table 1.

Table 1. The comparative *in vitro* susceptibilities of the *Candida albicans* complex strains isolated from patients with respiratory infection symptom to antifungal agents

Species n (%)	Antifungal	MIC_{50}	MIC_{90}	Range	Susceptible n (%)	Resistance n (%)	Dose Dependent n (%)
***C.albicans* 89.15%(74)**	fluconazole	1	32	0.125-64	56(75.67)	14 (18.91)	4 (5.4)
	itraconazole	0125	16	0.016-16	43(58.1)	19 (25.67)	12 (16.21)
	caspofungin	0.125	0.5	0.008-8	73(98.64)	1 (1.53)	--
	amphotericin B	2	8	0.016-16	28(37.83)	46 (62.16)	--
***C. dubliniensis* 9.63%(8)**	fluconazole	1	ND	0.5-64	3(37.5)	4 (50)	1 (12.5)
	itraconazole	0.125	ND	0.016-16	3(37.5)	4 (50)	1 (12.5)
	caspofungin	0.25	ND	0.016-1	(100)	--	--
	amphotericin B	2	ND	0.25-4	3(37.5)	5 (62.5)	--
***C. africana* 1.2%(1)**	fluconazole	ND	ND	0.5	1(100)	--	--
	itraconazole	ND	ND	0.016	1(100)	--	--
	caspofungin	ND	ND	0.008	1(100)	--	--
	amphotericin B	ND	ND	8	--	1 (100)	--

ND: not determined

Overall 18.91 % (n=14) of *C.albicans* isolates and 50 % (n=4) of *C. dubliniensis* were resistance to fluconazole (MIC≥64 µg/ ml). 25.67% (n=19) and 50 % (n=4) of *C.albicans* and *C. dubliniensis* were resistance to itraconazole respectively (MIC≥1 µg/ ml). For caspofungin, 1.53% (n=1) of *C.albicans* strain was resistant (MIC≥2 µg/ ml), and *C. dubliniensis* was susceptible at MIC ≤2µg/ ml. Our data were shown, 62.16 % (46) and 62.5 % (5) of *C.albicans* and *C. dubliniensis* were resistance to amphotricin B (MIC≥2 µg/ ml). *C.africana* was susceptible to fluconazole (MIC=0.5 µg/ml), itraconazole (MIC=0.016 µg/ml), Caspofungin (MIC=0.008 µg/ml), whereas resistance to amphotricin B (MIC=8 µg/ml).

Also our results indicated that, 5.4% (n=4) and 12.5 % (n=1) of *C. albicans* and *C.dubliniensis* were dose-dependent to fluconazole respectively (MIC=16-32µg/ ml), whereas 16.21 % (12) and 12.5 % (1) of these isolates were dose-dependent to itraconazole respectively (MIC=0.25–0.5µg/ml). Table 1 summarizes the results of MIC_{50} and MIC_{90}; *C.albicans* showed higher MIC_{50} (2µg/ml) with amphotericin B compared to caspofungin and azole tested agents. However, caspofungin demonstrated the lowest MIC_{50} (0.25 µg/ml) against *C.dubliniensis*. In addition, *C.africana* was fully susceptible to fluconazole (MIC=0.5 µg/ml), Itraconazole (MIC=0.016µg/ml), and caspofungin (MIC=0.008 µg/ml).

Results revealed, 8.91% of *C.albicans* were resistance to fluconazole and itraconazole. Besides, 16.2% of these isolates showed resistance to fluconazole, itraconazole, and amphotericin B.

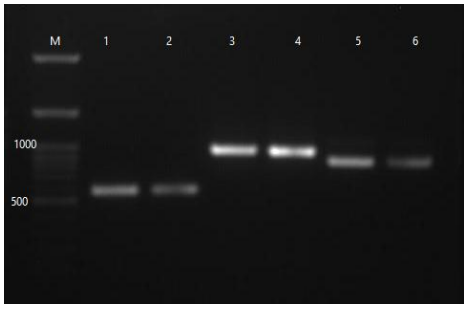

Figure 1. Species-specific amplification of the hwp1 gene; *C. dubliniensis* (569 bp) (lines:1,2), *C.albicans*(~1000 bp) (lines:3,4), and *C. africana* (~700 bp) (lines:5,6), using *hwp1* gene.

Discussion

C.albicans is the most common cause of invasive fungal infections and represents a serious public health challenge due to increasing medical and economic importance (14-16). However, *C.dubliniensis* emerging as a pathogen and has frequently been misidentified as *C.albicans* by clinical laboratories (7). In addition, *C.africana* which has been considered as a pathogen and reported as an atypical *C.albicans*, was isolated from patients suffered from vaginitis in several regions (8). Rapid identification of the mentioned strains seems to be crucial due to possible antifungal susceptibility as well as local epidemiological studies. In this investigation, the species that initially have been identified as *C.albicans* complex were further identified as *C.albicans* (89.15%), *C.dubliniensis* (9.63%), and *C.africana* (1.2%) by amplification of *hwp1* gene. Our results were different from the data reported by Bosco-Borgeat *et al.*, in which the rate of *C.dubliniensis* from fungemia was indicated as 0.96% (7). In addition, Moran *et al.*, have reported this rate as 2-3% of cases (5). Our obtained results shows the rate of *C.africana* to be 1.2% ($n_{=}1$) among all isolates, however this result was incompatible to the results of Romeo *et al.*, in which the mentioned rate was reported as 7.2% with the same method. The obtained antifungal susceptibility results indicated that *C.albicans* isolates from patients with respiratory infection symptoms were highly susceptible to caspofungin (98.64%) ($MIC_{90}\leq0.5$ µg/ml).This findings are in agreements with Lemos *et al.*, and Kumar *et al.*, (1,17). Resistance to the echinocandins was reported previously to be distinctly rare (overall range, 0.0 to 1.2%) among *C.albicans* (18,19). In this investigation 18.91% of *C.albicans* strains were highly resistance to fluconazole ($MIC\geq64$ µg/ ml). We also found that fluconazole had the widest range (0.0.063-64) and the highest MICs in *C.albicans*, whereas other studies like Wiebusch *et al.*, Wabe *et al.*, Njunda *et al.*, Shokohi *et al.*, Roy *et al.*, reported the rates of this resistance as 45.83%, 11.9%, 74.2%, 2.7%, 38.7% respectively (11,20-23) The difference in fluconazole resistance between our result and the same from Shokohi *et al.*, may be due to differences in investigated populations, as well as rare prescription of fluconazole to the most cancer patients as a standard care in Iran. Therefore, data exhibit a high prevalence of fluconazole resistance among *Candida* species may be correlated with the increased use of fluconazole in area of study. Other data reported by; Shokohi *et al.*, Al-mamari *et al.*, Aher *et al.*, Awari *et al.*, and Royet *et al.*, indicated the resistance of *C.albicans* to itraconazole as 5.4%, 10.3%, 36.9%, 35% and 19.3% respectively (20,24-26). However, 18.91% of *C.albicans* strains were shown to be fully resistant to both; itraconazole and fluconazole.

In addition, 62.16% of *C.albicans* isolates in our study were indicated to be resistant to amphotericin B. The latter result was similar to the result reported by Njunda *et al.*, (54.4%), and differ from the results of Aher *et al.*, (13.8%), Awari *et al.*, (7.5%) Roy *et al.*, *(*0%*)*, and Bosco-Borgeat *et al.*, (0%) (7,21,23,25-27). Obtained MIC Range (0.016-16) and MIC_{90} (8) for amphotericin B in present study differ from the data of Bosco-Borgeat *et al.*, which reported MIC Range and MIC_{90} as (0.13-1µg/ml) and (0.5) respectively (7). Besides, the rates 50%, 62.5%, and 62.5% of *C.dubliniensis* strains were resistance to fluconazole, itraconazole, and amphotericin B respectively. However, 100% of strains were susceptible to caspofungin. Compared with the results of Bosco-Borgeat *et al.*, our result showed lower susceptibility of fluconazole and itraconazole in *C.albicans* and *C. dubliniensis* strains. Such incompatibility is also observed with the results of Zhang *et al.*, that reports the resistance to fluconazole in 20% of *C.dubliniensis* (27). According to our data, *C.africana* was susceptible to fluconazole, itraconazole, caspofungin and resistance to amphotericin B. Caspofungin demonstrated the highest activity against *C.albicans, C.dubliniensis* and *C.africana*. Therefore caspofungin seems to be the most active drug for pulmonary and disseminated candidiasis treatment (28). In conclusion, this study revealed, antifungal susceptibility pattern in *C.albicans, C.dubliniensis* and *C.africana* derived from clinical samples of patient with respiratory infection symptoms. Such a local surveillance studies is required for control the spread of resistance which would be helpful to develop effective

treatment strategies, prophylaxis and antifungal susceptibility pattern of different *Candida* strains that seems to be important for appropriate therapy.

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