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Antifungal Susceptibility Profiles of *Candida* Species Isolated from Ahvaz Jundishapur Educational Hospitals

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Abstract

Background: *Candida* infections are one of the most important nosocomial infections that have increased by 3.5 to 14 folds over the past decades. Although the sources of infection are human normal flora, hospital environments have an undeniable role. The increased use of antifungals, prolonged prophylaxis, and some organism-associated genetic factors have led to antifungal resistance.
Objectives: The aim of the present prospective study was to identify *Candida* species from clinical specimens, normal flora, and hospital environments. Furthermore, the susceptibility profile of strains to several antifungals was also evaluated.
Methods: Two hundred and twenty-one samples (clinical specimens, hospital environments, and personal normal flora) were collected. Samples were inoculated on CHROMagar *Candida*, incubated at 35°C, and were identified using classical and molecular techniques. Consequently, all recovered isolates were tested against six antifungal drugs, using the microdilution method.
Results: Ninety-two *Candida* strains, belonging to 10 different yeast species, were detected with the most common isolate, *Candida albicans* (46.74%). *Candida albicans* made up the majority of species that were obtained from oral samples and non-*albicans* species with uncommon frequency were obtained from hospital environment samples. Miconazole was a unique antifungal, towards which all strains were sensitive. However, most of the isolates were also sensitive to fluconazole.
Conclusions: Although resistance to amphotericin B, terbinafine, fluconazole, caspofungin, and itraconazole was found among *C. albicans* and non-*albicans* species, however, miconazole is the most effective antifungals against all strains.

Keywords: Antifungals Susceptibility, Hospital Environment, PCR-RFLP, *Candida* Species

1. Background

Hospital environments are inevitably great sources of opportunistic fungal pathogens, which may be transmitted to both inpatients and outpatients by different routes. Nosocomial (hospital acquired) infections commonly occur during hospitalization in specialized wards, including urology, surgery, intensive care units (ICUs), neonatal intensive care units (NICUs), and infectious diseases among immunocompromised patients (1-4). Nosocomial infections have been increased dramatically in the past two to three decades due to several risk factors, including organ transplantation, hospitalization at ICUs and NICUs, malignancy, chemotherapy, and immunosuppression (2, 5). Furthermore, yeast infections, including candidiasis, have also been increased from 6 to > 10% during the recent decades (6).

Infections by members of the genus *Candida* mainly have an endogenous origin. However, candidiasis with exogenous sources are generally a cross-infection that is transmitted via the health care staff hands or relatives, patient to patient, or even by medical devices (6-8). Candidemia is a serious infection with significant mortality, mainly caused by *Candida* species. Therefore, candidiasis is known as the fourth cause of septicemias in the US and the sixth to tenth in Europe (9, 10). Studies have shown that a 3.5- to 14-fold increase was observed in *Candida* infections over the past two decades, especially during hospitalization at ICUs and NICUs (11). Accordingly, morbidity and mortality was increased among nosocomial infections due to *Candida* species (12).

Approximately, 66% to 80% of fungal infections include different forms of candidiasis. The causative agents of 70% to 80% are *Candida albicans* strains and the rest are non-*albicans* species (6, 13). In total, the incidence of invasive candidiasis is 7 to 10 times higher than invasive aspergillosis (9). However, invasive candidiasis occurs

only in 1% to 8% of hospitalized patients (14). The rate of transmission of the *Candida* species varied from 0% to 58% among health care personnel (13). Furthermore, an increase in infections associated with medical equipment was detected. For example, at least 50% of nosocomial infections are associated with medical equipment, especially septicaemia and urinary tract infections (8). Recently, authors reported that intravenous catheter administration and hemodialysis are responsible for 87% of primary blood infections due to non-*albicans* species (8, 10).

In the recent years, there has been a shift towards invasive candidiasis due to non-*albicans* species. This shift is due to the use of antifungals (azoles), prolonged prophylaxis, and genetic factors (9, 15-17). *Candida krusei* is inherently resistant to some antifungals (12) and 3% to 7% of *C. glabrata* are resistant to fluconazole (18).

2. Objectives

The aim of the present prospective study was to determine the frequency of *Candida* species isolated from different hospital environments, clinical samples, and staffs normal flora. Furthermore, the susceptibility profile of isolates to antifungals, such as fluconazole, amphotericin B, terbinafine, itraconazole, miconazole, and caspofungin was investigated.

3. Methods

3.1. Ethics Statement

The present study protocol was reviewed and approved by the Ethical and Research Committee of Ahvaz Jundishapur University of Medical Sciences (ethic number: IR.AJUMS.REC.1395.65).

3.2. Sampling and Isolation

In the present prospective study, 221 samples were randomly collected from different ward [surgery, infectious disease, Neonatal intensive care unit (NICU), nephrology, intensive care unit (ICU)] environments of educational hospitals (Golestan, Abuzar, Imam Khomeini, and Razi Hospitals) of Ahvaz, Iran during April to July 2016. Seventy-one (32.1%) of the samples were collected from clinical samples including, urines, stools, and respiratory tracts secretions. Furthermore, 72 (32.6%) of the samples were randomly obtained from oral cavities, neonates skin, nurses, and physician's hands. Moreover, 78 (35.3%) samples were collected from hospital environments, lab coats, and different instruments in wards. All samples were cultured on CHROM agar Candida (CHROMagar Candida, France) and incubated at 37°C for 48 hours. All strains were subcultured on Sabouraud dextrose agar (BioLife, Italia) and preserved at low temperature for further studies.

3.3. Primary Identification

The primarily identification was based on morphological and microscopy characteristics, including colonies coloration on CHROM agar Candida and growth at 42 to 45°C as well as germ tube formation and micromorphology on cornmeal agar (Difco, USA), supplemented by 1% Tween 80 (Merck, Germany).

3.4. Identification of Isolates

All isolates were confirmed using a molecular technique, PCR-RFLP method, according to Mohammadi et al. (19). Firstly, the genomic DNA of each isolate was extracted by boiling of a loopful of fresh yeast colony suspended in 100 μL of deionized distilled water and heated at 100°C for 10 minutes, as previously described. The suspensions were then centrifuged at 4000 g for 10 minutes and kept at -20, as a DNA template. The ITS1-5.8S-ITS2 fragment of r-DNA complex was amplified in all strains using ITS1/ITS4 primer pair (20). The amplified products were digested with the restriction enzymes *Msp*I in a 30-μL reaction mixture, according to the manufacturer's instructions. Finally, the digested fragments were separated through electrophoresis on 2% gel agarose, stained with ethidium bromide, visualized under UV light and photographed. For identification of isolates, the banding pattern of each strain was compared with the banding profile described in a previous study (19).

3.5. Antifungals Stock and Working Solutions

A stock solution of caspofungin (Sigma-Aldrich, Germany) 1.25 mg/mL, itraconazole 2.5 mg/mL (Sigma-Aldrich, Germany), amphotericin B (Sigma-Aldrich, Germany), fluconazole (Serva, USA), terbinafine (Sigma-Aldrich, Germany), miconazole (Sigma-Aldrich, Germany) 32 mg/mL, was prepared in dimethyl sulfoxide (DMSO, Fluka, Germany). Stock solutions were kept at room temperature for complete dissolving and then stored at -20°C until use. A serial dilution of antifungal was prepared from 2 to 0.016 μg/mL for caspofungin, 16 to 0.125 μg/mL for itraconazole, 16 to 0.125 μg/mL for amphotericin B, 32 to 0.25 μg/mL for fluconazole, 128 to 1 μg/mL for terbinafine, and 4 to 0.031 μg/mL for miconazole (21).

3.6. Antifungal Assay

A standard suspension of an overnight culture of all tested organisms was prepared in 0.01% Resazurin (Sigma - Aldrich, Germany) RPMI 1640 (Bio IDEA, Iran), according to the CLSI protocol (21, 22). Then, 100 μL of suspension and 100 μL of antifungal serial dilutions were added to each well of a 96-well microplate. Microplates were incubated at 35°C for 24 to 48 hours, then the MIC range, MIC_{50}, MIC_{90}, and MIC_{GM} were calculated.

Breakpoints were set by CLSI for azoles as follow; itraconazole (susceptible, MIC < 1 μg/ mL; dose dependent, 0.25 to 0.5; resistant, MIC $\geq$ 1 μg/mL), and fluconazole (susceptible, MIC $\leq$ 2 μg/mL; sensitive dose dependent, 4 μg/mL; resistant, MIC $\geq$ 8 μg/mL). There was no defined breakpoint for miconazole, however, in literature, it is indicated that *Candida* is susceptible and resistant at MIC $\leq$ 5 μg/mL and MIC > 5 μg/mL, respectively. Moreover, *C. glabrata*, *C. parapsilosis*, and *C. albicans* are sensitive (S) to caspofungin at MIC $\leq$ 0.12, MIC $\leq$ 0.2, and MIC $\leq$ 0.25 μg/mL, respectively. However, resistant ranges for them are MIC $\geq$ 0.5, MIC $\geq$ 0.8, and MIC $\geq$ 1 μg/mL, respectively. Terbinafine susceptibility breakpoints are $\leq$ 8 μg/mL susceptible and > 8 μg/mL resistant. Although a defined breakpoint is unavailable for amphotericin B, MICs $\leq$ 1 and >2 mg/mL were considered as susceptible and resistant, respectively (23-33).

4. Results

Out of 221 collected samples from clinical materials, hospital environments and normal skins, 70 (31.7%) cases yielded *Candida* species (Table 1). The study indicated that 10.3% (8 of 78 samples) of hospitals environments, 49.3% (35 of 71 samples) of clinical samples (stools, urines, and respiratory tracts samples), and 37.5% (27 of 72 samples) of normal flora (staffs hands, neonates skins and oral cavity) were contaminated to different species of *Candida*. The most common recovered *Candida* species was *C. albicans* 43 (46.7%), followed by *C. glabrata* (21, 22.8%), *C. tropicalis* (12, 13.0%), *C. parapsilosis* (6, 6.5%), *C. krusei* (3, 3.3%), *C. rugosa* and *C. famata* (each one 2, 2.2%), *C. kefyr, C. lusitaniae*, and *C. guilliermondii* (each one 1, 1.1%). In the present study, in 22 (31.4%) cases multispecies of *Candida* were found (Table 1).

The results of *in vitro* antifungal susceptibility profiles (MIC range, MIC_{50}, MIC_{90} and MIC_{GM}) of six antifungals against all *Candida* species are shown in Table 2. Although all strains were sensitive to miconazole, only 5 and 10 isolates of *C. albicans* were resistant to fluconazole and caspofungin, respectively. The results showed that 16 isolates of *C. albicans* were resistant to itraconazole, followed by one isolate of *C. glabrata*, five isolates of *C. tropicalis*, and one isolate of *C. parapsilosis*. Furthermore, 29 isolates of *C. albicans* and three isolates of *C. tropicalis* were resistant to terbinafine. As shown, this study found that 30 isolates of *C. albicans*, two isolates of *C. glabrata*, seven isolates of *C. tropicalis*, and three isolates of *C. parapsilosis* were amphotericine B-resistant.

The MIC range for rare non-*albicans* species of *C. krusei*, *C. kefir*, *C. lusitaniae*, *C. guilliermondii*, *C. rugosa*, and *C. famata* is shown in Table 3. As shown, only one isolate of *C. famata* was resistant to amphotericin B after 48 hours of incubation. In addition, resistance to terbinafine was observed in *C. guilliermondii*, and one isolate of *C. rugosa*. In the present study, several multi-resistance was observed among tested *Candida* species (Table 4).

5. Discussion

Candida species are human mycobiota and are considered as an important opportunistic pathogen causing life-threatening diseases, especially in patients with immunodeficiency. Furthermore, *Candida* species have been identified as the common cause of nosocomial infection (34). Moreover, the frequency of nosocomial infections due to *Candida* species have been increased worldwide with a high rate of morbidity and mortality (35).

Various studies have shown that hospital environments and staff hands as well as medical devices are contaminated with fungal agents. In a study by Savastano et al. 19.65% of collected samples from environmental health practitioners of a Brazilian hospital were contaminated with different species of *Candida* (35). In a similar study, Storti et al. found that 19.2% of theirs samples from inpatients, the environment, and health practitioners yielded *Candida* species (5). Although the total frequency of *Candida* in the current study was 31.7%, only 10.3% of hospital environments were contaminated with *Candida* species. On the other hand, this study only isolated *Candida* from one case of staff hands and two cases of neonate skins (7.5%). Furthermore, 57.3% of stools, urines, swab from oral cavity, and respiratory tract samples had positive cultures. It is believed that the hospital environments have different mycoflora and usually spread via staff hands (36, 37). In addition, moist surfaces in hospitals protect *Candida* species for a long time (38).

In the present study, *C. albicans* was the most common isolate with a frequency of 46.7%, followed by *C. glabrata* (22.8%), *C. tropicalis* (13.0%), *C. parapsilosis* (6.5%), *C. krusei* (3.3%), *C. rugosa* (2.2%), *C. famata* (2.2%), *C. kefyr* (1.1%), *C. lusitaniae* (1.1%), and *C. guilliermondii* (1.1%). *Candida albicans* was predominantly isolated from clinical samples, whereas

Table 1. Distributions of *Candida* Species Isolates from Clinical and Environmental Samples[a]

Samples Sites	Total Samples[a]	Positive Cases[a]	*Candida* Species										
			C. p	C. a	C. g	C. t	C. k	C. r	C. ke	C. f	C. gu	C. l	Total
Staffs hands	34 (15.4)	1 (1.4)	1										1
Neonates skin	6 (2.7)	2 (2.9)	1	1									2
Oral cavies	32 (14.5)	24 (34.3)		24	9	2			1				36[b]
Urines	44 (19.9)	22 (31.4)	1	7	5	7	3	2					25[b]
Stools	5 (2.3)	5 (7.1)		3	4								7[b]
Respiratory tracts	22 (10.0)	8 (11.4)		7	3								10[b]
Hospitals Environments	78 (35.3)	8 (11.4)	3	1		3				2	1	1	11[b]
Total	221 (100)	70 (100)	6	43	21	12	3	2	1	2	1	1	92

Abbreviations: C. a, *C. albicans*; C. f, *C. famata*; C. g, *C. glabrata*; C. gu, *C. guilliermondii*; C. l, *C. lusitaniae*; C. k, *C. krusei*; C. ke, *C. kefir*; C. p, *Candida parapsilosis*; C. r, *C. rugosa*; C. t, *C. tropicalis*.
[a]Values are expressed as No. (%).
[b]Multispecies.

both *C. tropicalis* and *C. parapsilosis* were mainly isolated from environmental materials. *Candida glabrata* (37.6%) was more frequently isolated from the environment, followed by *C. parapsilosis* (25.74%) and *C. tropicalis* (16.83%) in Savastano et al.'s study (35). In another study by Storti et al., only one isolate of *C. albicans* was isolated from 270 environmental and clinical samples taken from hospital and the rest of them (51 cases) were non-*albicans*, including *C. tropicalis*, *C. guilliermondii*, *C. parapsilosis*, *C. lusitaniae*, and *C. krusei* (5). Similar to the current study, in Sabino et al.'s report, *C. parapsilosis* strains were the most abundant isolates from the hospital environment. Furthermore, they believe that these isolates were more pathogenic than clinical isolates (39).

The sensitivity pattern of *Candida* species to antifungals is a powerful tool for clinicians to better use a prophylactic, pre-emptive, and empiric antifungals therapy. On the other hand, prophylactic and empirical uses of azole derivatives have increased the frequency of non-*albicans* *Candida* species in hospitals (40, 41). In the current study, all of isolates were only susceptible to miconazole antifungals. Miconazole was effective against all tested *Candida* isolates, including fluconazole resistance strains in Isham and Ghannoum study (42). Furthermore, all *C. albicans* and non-*albicans* species in Storti's study were sensitive to fluconazole (5). In contrast, a resistance to miconazole and fluconazole up to 33.3% and 50% in non-*albicans* *Candida* was observed in Savastano et al.'s study (35). The current isolates were a mixture of clinical, environmental, and resistant strains to fluconazole, found among 11.6% of *C.albicans*, similar to 10.5% of tested *C. albicans* by Badiee and Alborzi (43).

Caspofungin is a new antifungal with broad spectrum against mold and yeast fungi and there are a few cases of caspofungin-resistance among *Candida* species. Pfaller et al. reported only 0.1% resistance to caspofungin in 5346 isolates of *Candida* (44). However, Baghdadi et al. (34) and Amanloo et al. (45) did not find any isolate to be resistant to caspofungin. In contrast, this study found that 15 isolates of *Candida* species were resistant to caspofungin. In a previous study by Rezaei-Matehkolaei et al. only one clinical isolate of *C. albicans* was resistant to caspofungin (25). However, 4.6% of tested isolates of *C. albicans* by Shokohi et al. were resistant to caspofungin (46). This study observed that there are considerable levels of resistance against amphotericin B, followed by terbinafine and itraconazole. The susceptibility of *Candida* to itraconazole varied in the current report. Non-*albicans* *Candida* species were resistant to itraconazole up 33.3% in Savastano et al.'s report (35), in contrast, all strains of *Candida* collected by Bonfietti et al. were sensitive to itraconazole (47). The author's previous study showed that *C. albicans* (seven isolates) and *C. parapsilosis* (two isolates) from clinical specimens were resistant to amphotericin B (48).

5.1. Conclusions

Candida albicans was the major species that was obtained from oral samples and non-*albicans* species with uncommon frequency were obtained from hospital environmental samples. Although resistance to amphotericin B, terbinafine, itraconazole, caspofungin, and fluconazole was found among *C.albicans* and non-*albicans* species, miconazole is an effective antifungal against all strains.

Table 2. *In Vitro* Susceptibilities of *Candida* spp. to Antifungal Agents, MIC Range, MIC_{50}, MIC_{90} and MIC_{GM} After 24 Hours

Antifungals	MIC Ranges	Minimum Inhibitory Concentration, μg/mL			Resistant[a]	
		MIC50	MIC90	MICGM	CLSI M27-A3	CLSI M27-S4
Candida albicans **(n = 43)**						
Fluconazole	< 0.25 - 32	0.5	16	0.636	0 (0)	5 (11.6)
Amphotericin B	< 0.125 - >16	16	> 16	3.75	30 (69.8)	-
Terbinafine	< 1 - > 128	128	> 128	30.98	29 (67.4)	-
Itraconazole	< 0.125 - 4	0.125	2	0.27	16 (37.2)	-
Miconazole	< 0.031 - 0.5	0.062	0.25	0.054	0 (0)	-
Caspofungin	< 0.015 - 2	0.25	1	0.18	0 (0)	10 (23.3)
Candida glabrata **(n = 21)**						
Fluconazole	< 0.25 - 0.5	< 0.25	< 0.25	0.133	0 (0)	-
Amphotericin B	< 0.125 - 16	< 0.125	< 0.125	0.10	2 (9.5)	-
Terbinafine	< 1	< 1	< 1	0.5	0 (0)	-
Itraconazole	< 0.125 - 2	< 0.125	< 0.125	0.073	1 (4.8)	-
Miconazole	< 0.031 - 0.5	< 0.031	< 0.031	0.023	0 (0)	-
Caspofungin	< 0.015 - 1	< 0.015	< 0.015	0.009	0 (0)	1 (4.8)
Candida tropicalis **(n = 12)**						
Fluconazole	< 0.25 - 2	< 0.25	2	0.31	0 (0)	0 (0)
Amphotericin B	< 0.125 - 16	2	16	1.58	7 (58.3)	-
Terbinafine	< 1 - 128	< 1	128	2	3 (25)	-
Itraconazole	< 0.125 - 4	0.5	2	0.33	5 (41.7)	-
Miconazole	< 0.031 - 0.5	0.125	0.25	0.088	0 (0)	-
Caspofungin	< 0.015 - 1	0.125	1	0.23	0 (0)	4 (33.3)
Candida parapsilosis **(n = 6)**						
Fluconazole	< 0.25	-	-	-	0 (0)	0 (0)
Amphotericin B	< 0.125 - 8	-	-	-	3 (50)	-
Terbinafine	< 1 - 1	-	-	-	0 (0)	-
Itraconazole	< 0.125 – 1	-	-	-	1 (16.7)	-
Miconazole	< 0.031	-	-	-	0 (0)	-
Caspofungin	< 0.015 - 1	-	-	-	0 (0)	0 (0)

[a] Values are expressed as No. (%).

Table 3. The MIC Range of the Rare *Candida* Species to Antifungal Agents After 24 Hours

Candida Species	MIC Range, μg/mL					
	Flu	Amp	Cas	Itr	Ter	Mic
Candida krusei **(n = 3)**	< 0.25	< 0.125	< 0.015 - 0.25	< 0.125	< 1	< 0.031
C. rugosa **(n = 2)**	< 0.25	< 0.125	0.5 - 1	< 0.125	< 1 - 64	< 0.031
C. famata **(n = 2)**	< 0.25	< 0.125 - 1	< 0.015	< 0.125	< 1	< 0.031
C. kefyr **(n = 1)**	< 0.25	< 0.125	< 0.015	< 0.125	< 1	< 0.031
C. lusitaniae **(n = 1)**	< 0.25	< 0.125	< 0.015	< 0.125	< 1	< 0.031
C. guilliermondii **(n = 1)**	< 0.25	< 0.125	< 0.015	< 0.125	128	< 0.031

Abbreviations: Amp, amphotericin B; Cas, caspofungin; Flu, fluconazole; Itr, itraconazole; Mic, miconazole; Ter, terbinafine.

Footnotes

Authors' Contribution: Study concept and design, Ali Zarei Mahmoudabadi and Ali Rezaei-Matehkolaei; conducting the experiments, Simin Taghipour; data analysis and interpretation of results: Simin Taghipour, Ali Zarei Mahmoudabadi and Ali Rezaei-Matehkolaei; drafting of

Table 4 . Multi-Resistance Against Amphotericin B, Itraconazole, Terbinafine, Fluconazole, Terbinafine and Caspofungin

Candida Species	Multi Resistant					
	CLSI M27-A3			CLSI M27-S4		
	Am B	Itra	Ter	Flu	Ter	Cas
C. albicans **(15 isolates)**	R	R	R			
C. albicans **(4 isolates)**				R	R	
C. albicans **(2 isolates)**				R	R	R
C. parapsilosis **(1 isolate)**	R	R				
C. glabrata **(1 isolate)**	R	R				
C. tropicalis **(1 isolate)**	R	R	R			
C. tropicalis **(1 isolate)**	R	R				

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Conflict of Interests: The authors are responsible for the content and the writing of the paper.

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