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Multicenter Identification and Antifungal Susceptibility Patterns of *Candida* Species Isolated from Clinical Samples

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Abstract

Background: Invasive fungal infections without proper treatment could lead to high mortality rate, especially in immunocompromised patients. *Candida* species distribution and drug susceptibility patterns vary in different areas. Understanding the etiologic agents and drug susceptibility patterns in each region are required for the best management of patients with *Candida* infections.
Objectives: The aim of this study was to identify *Candida* species isolated from clinical samples of six university hospitals in Iran and detect their susceptibility patterns to seven antifungal agents.
Methods: Clinical samples from patients with fungal infections were cultured on Sabouraud dextrose agar. Isolated yeasts were identified by API 20C AUX kit, according to the manufacturer's instructions. Drug susceptibility patterns to amphotericin B, caspofungin, voriconazole, fluconazole, posaconazole, itraconazole and ketoconazole were determined, according to CLSI M27-A3 and S4.
Results: In total, 428 species of *Candida* were isolated from clinical samples (1950 samples). Most isolated species were *Candida albicans*, followed by *C. tropicalis*, *C. parapsilosis*, *C. kefyr, C. famata, C. glabrata, C. krusei, C. dubliniensis, C. guilliermondii* and *C. lusitaniae*. Sensitivity rate of *C. albicans* to amphotericin B, caspofungin, voriconazole, fluconazole, and itraconazole was 96.6%, 99.5%, 88.6%, 90.6%, and 52% with MIC90 values equal to 0.25 μg/mL, 0.125 μg/mL, 0.125 μg/mL, 2 μg/mL, and 1 μg/mL, respectively. The MIC 90 values for ketoconazole and posaconazole were 0.125 μg/mL and 0.064 μg/mL, respectively. Different sensitivity to antifungal agents was present in *non-albicans Candida* species especially in *C. krusei, C. glabrata*, and *C. tropicalis*.
Conclusions: According to this study, *C. albicans* is the most prevalent etiologic agent in infected patients and caspofungin is the most effective antifungal agent. Knowledge about etiologic agents and their susceptibility patterns in each region is helpful for successful treatment of the patients.

Keywords: *Candida albicans*, Fluconazole, Amphotericin B, Itraconazole, *Candida tropicalis*

1. Background

Systemic candidiasis in immunocompromised patients is associated with high morbidity and mortality rates, especially in those not responsive to antifungals (1, 2). *Candida albicans* is the most prevalent isolate from human infections, however other *Candida* species have been reported as well (3, 4). The relative frequency of non-*albicans Candida* species varies in different areas, for example, in North America *C. glabrata* is the second and in Iran is the third species isolated after *C. albicans* (5, 6). The epidemiology of candidemia varies, according to geographical region. Also, a variety in the distribution of *Candida* species in different areas was reported (2-6). The reason of emerging non-*albicans* species is not clear yet and can be associated with improvements in more sensitive methods for the identification of *Candida* species and indiscriminate use of antifungal drugs for prophylaxis and treatment (7, 8).

In the recent years, treatments of systemic candidiasis are a challenge due to resistant etiologic agents and the emergence of infections caused by species other than *C. al-*

bicans (3, 5, 6). Adequate knowledge about the etiologic agents and drug susceptibility patterns are required for treating the hospitalized patients with risk factors of systemic candidiasis.

2. Objectives

This study aimed at identifying *Candida* species isolated from clinical samples in six university hospitals (Shiraz, Isfahan, Tehran, Urmia, Sari, and Mashhad) and determined their susceptibility patterns to seven antifungal agents.

3. Methods

3.1. Isolates Sources

Clinical samples (1950 samples) including abscess, wound, blood, cerebrospinal fluid, bronchoalveolar lavage, vaginal discharge, nails, and sputum were sent to mycology laboratories of tertiary hospitals in Shiraz, Isfahan, Tehran, Urmia, Sari, and Mashhad. They were cultured on Sabouraud dextrose agar (Merck, Germany), containing chloramphenicol (Merck, Germany), according to standard protocols related to each sample and incubated for 48 hours at room temperature for rapidly growing species and up to seven days for slow growing. All *Candida* species isolated from hospitalized patients with signs and symptoms of fungal infections were entered in this study. Isolated *Candida* species were transferred to Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, and identified by API 20C AUX kit (BioMerieux, France), according to the manufacturer's protocol.

3.2. Antifungal

Antifungal susceptibility test to amphotericin B (AMB, Sigma-Aldrich, Germany), caspofungin (CAS, Sigma-Aldrich, USA), voriconazole (VRC, Sigma-Aldrich, USA), fluconazole (FLU, Sigma-Aldrich, USA), posaconazole (POS, Sigma-Aldrich, Germany), itraconazole (ITR, Sigma-Aldrich, India), and ketoconazole (KET, Sigma-Aldrich, China) was performed according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 and CLSI M27-S4 (9, 10). Briefly, RPMI medium with L-Glutamine and without sodium bicarbonate (Sigma, Germany) was buffered with 0.165 mole per liter of 3-(N-Morpholino) propanesulfonic acid, 4 morpholinepropanesulfonic acid (Sigma, Germany). pH was adjusted to 7 and filtered in sterile conditions.

3.3. Susceptibility Tests

Stock concentration of each drug was prepared in a suitable solvent (water for CAS and DMSO (Merck, Germany) for other drugs) and diluted with RPMI to obtain the working concentration solution. Serial dilutions from AMB, CAS, VOR, POS, ITR, and KET, ranging from 0.032 to 16 μg/mL and for FLU from 0.125 to 64 μg/mL, were prepared. For each series, positive (well without antifungal) and negative (well without yeast) controls were considered. In a 96 - well plate (JETBIOFIL, China), 100 μL of RPMI and serial dilutions were poured in each well. The suspensions with a concentration equal to 0.5 McFarland were prepared from 24 - to 48 - hour incubated colonies (1×10^6 to 5×10^6 cells/mL) and diluted at 1:1000 with RPMI (1×10^3 to 5×10^3 cells/mL). Yeast suspensions (100 μL) were added to each well, except the control negative well. The final yeast concentration in each well was 0.5×10^3 to 2.5×10^3 cells/mL. Plates were kept at 35°C for 24 to 48 hours and were read visually after the incubation time. Minimum Inhibitory Concentrations (MIC) for CAS, VOR, FLU, POS, ITR, and KET were described as the lowest concentration of the drug that could reduce fungal growth by 50% to 80%, compared to positive controls. For AMB, MIC was described as the lowest concentration of the drug that could stop any visible yeast growth.

3.4. Statistical Analysis

Data were analyzed using the WHONET 5.6 software, and MIC50, MIC90 (MIC values which inhibit 50% and 90% of the isolates) and geometric mean was calculated for each drug and each isolate. New *Candida* species breakpoint provided by the CLSI was used in the present study. These new breakpoints are drug and species specific; POS and KET have no breakpoint in the new CLSI (10).

4. Results

Overall, 428 *Candida* were isolated from clinical samples, the prevalent species was *C. albicans* (273, 63.78%), followed by *C. tropicalis* (38, 8.87%), *C. parapsilosis* (35, 8.17%), *C. kefyr* (20, 4.67%), *C. famata* (20, 4.67%), *C. glabrata*, (18, 4.2%), *C. krusei* (10, 2.34%), and other species (14, 3.27%). The second most frequent isolate from Shiraz, Mashhad, and Sari was *C. tropicalis* while *C. parapsilosis* in Isfahan and Tehran, and *C. famata* in Urmia were the second most frequently isolated. Distributions of the isolated species are presented in Table 1.

Susceptibility patterns of the common *Candida* species isolates are shown in Table 2 and the uncommon *Candida* species isolates are shown in Table 3. The sensitivity rates of *C. albicans* species to AMB, CAS, VRC, and FLU were

Table 1. Distribution of *Candida* Species Isolated From Six University Hospitals in Iran

Species	Shiraz	Mashhad	Isfahan	Urmia	Tehran	Sari	Total[a]
Candida albicans	47	52	55	38	41	40	273 (63.78)
Candida tropicalis	7	6	9	5	4	7	38 (8.87)
Candida parapsilosis	3	5	10	6	7	4	35 (8.17)
Candida kefyr	3	2	5	4	3	3	20 (4.67)
Candida famata	2	2	-	9	5	2	20 (4.67)
Candida glabrata	3	4	6	1	2	2	18 (4.20)
Candida krusei	5	1	3	-	1	-	10 (2.34)
Others[b]	1	1	4	4	2	2	14 (3.27)
total	71	73	92	67	65	60	428

[a] Data are presented as No (%)
[b] *Candida dubliniensis*, *Candida guilliermondii* and *Candida lusitaniae*

96.6% (271.9/273), 99.5% (272/273), 88.6% (242/273), and 88.3% (241/273), respectively. The resistance rate of *C. albicans* to ITR was 12.7% with susceptible dose dependence of 35.3% and sensitivity rate of 52%. The MIC90 values and geometric means for POS and KET in this species were 0.064 μg/mL and 0.027 μg/mL, and 0.125 μg/mL and 0.028 μg/mL, respectively.

Candida tropicalis was the second most isolated species from patients; it was more sensitive to CAS, POS and KET with MIC90 value of 0.125 μg/mL. The MIC90 value of *C. glabrata* for AMB and CAS, VRC, FLU, POS, ITR and KET was 4 μg/mL, 2 μg/mL, 2 μg/mL, 16 μg/mL, 2 μg/ml, 4 μg/mL, and 2 μg/mL, respectively. The resistance rates of *C. guilliermondii* to both AMB and ITR were 20%, with MIC90 value of 16 μg/mL for KET.

The most resistance rate among isolated species to AMB and CAS was in *C. krusei* (MIC90 8 and 2 μg/mL, respectively). *Candida krusei* sensitivity rates to AMB, VOR and ITR were 70% (7/10), 50% (5/10), and 33.3% (3/10), respectively. *Candida parapsilosis* was completely sensitive to all antifungal agents and susceptible dose dependent rate to ITR was 60%. *Candida kefyr* was sensitive to all the antifungal drugs and 10% (2/20) of *C. famata* were resistant to ITR and 5% (1/20) resistant to AMB.

5. Discussion

Candida species could cause severe infections with high morbidity and mortality in hospitalized patients (2-4). *Candida albicans* was reported as the most isolated species from infected patients in this study and other studies in Asian, European, and American countries (3, 5, 11-14). A global increase was seen in the number of infections caused by species other than *C. albicans*, including *C. tropicalis, C. parapsilosis, C. glabrata*, and *C. krusei*(8, 15). *Candida tropicalis* was the second isolated *Candida* species in this study. The epidemiology of this species was reported as 15.3% in Brazil(16), 13.4% in Iran (17), and 8.4% in Italy (18).

Amphotericin B is a polyene antifungal drug with high activity against a wide variety of fungal pathogens. The sensitivity rates of *C. albicans* to AMB in this study was 96.6% (271.9/273), and in other studies, this was reported as 97.4% (37/38), 96.6% (113/117), 100% (93/93), and 93% (160/172) (11, 15, 19, 20). The sensitivity rate of *C. glabrata* in this study was 88.9% (16/18) and in other studies, this was reported as 85% (34/40) and 93.8% (15/16) (6, 11). This rate for *C. krusei* was 70% (7/10) in the present study and 90% (56/62) in the other studies (6). The differences in sensitivity rates could be due to patient population or type of the study. Given the limited number of isolates in non-*albicans* species, high resistant rate may not be reliable enough.

Echinocandins are a new line of antifungal drugs. According to Espinel-Ingrof et al. (2013), use of the CLSI species-specific CAS breakpoint could lead to results indicating an excessive number of sensitive isolates (such as *C. glabrata* and *C. krusei*) as resistant. Therefore, they suggested that routine testing or reporting of CLSI CAS MICs for *Candida*, according to CLSI (2012), is not suitable (21). Generally, in this study, CAS was an effective agent against *Candida* species, except some *C. glabrata* and *C. krusei* species (MIC90 = 2 μg/mL). The MIC90 values of this drug for *C. glabrata* and *C. krusei* were reported as 0.19 and 0.75 μg/mL in colonized isolates in 2014 (15); 4 and 1 μg/mL in patients hospitalized at ICUs and urology wards (22); and 0.125 and 0.25 μg/mL in patients with cancer (11). In this study, all isolates were from infected patients and this is the reason for the differentiation between the studies.

Table 2. Sensitivity Pattern of Common *Candida* Species Isolated From Clinical Samples in Six University Hospitals of Iran

Candida	Antibiotic Name	Breakpoints	%R[a]	%I[b]	%S[c]	MIC50, μg/mL	MIC9, μg/mL	MIC Range, μg/mL
Candida albicans	Amphotericin B	S ≤ 1, R ≥ 1	3.4	0	96.6	0.032	0.25	0.032 - 16
	Caspofungin	S ≤ 0.25, I = 0.5, R ≥ 1	0.5	0	99.5	0.032	0.125	0.032 - 1
	Voriconazole	S ≤ 0.12, I = 0.25, - 0.5 R ≥ 1	6.9	4.5	88.6	0.032	0.125	0.032 - 2
	Fluconazole	S ≤ 2, SDD = 4, R ≥ 8	4.9	4.5	90.6	0.125	2	0.032 - 64
	Posaconazole	WBP[d]	0	0	0	0.032	0.064	0.01 - 1
	Itraconazole	S ≤ 0.12, SDD = 0.25, - 0.5 R ≥ 1	12.7	35.3	52	0.064	1	0.032 - 2
	Ketoconazole	WBP	0	0	0	0.032	0.125	0.01 - 1
Candida tropicalis	Amphotericin B	S ≤ 1, R ≥ 1	19	0	81	0.064	4	0.032 - 8
	Caspofungin	S ≤ 0.25, I = 0.5, R ≥ 1	0	4.8	95.2	0.032	0.125	0.032 - 0.5
	Voriconazole	S ≤ 0.12, I = 0.25, - 0.5 R ≥ 1	14.3	14.3	71.4	0.032	1	0.032 - 16
	Fluconazole	S ≤ 2, SDD = 4, R ≥ 8	9.5	0	90.5	0.25	2	0.032 - 64
	Posaconazole	WBP	0	0	0	0.032	0.125	0.032 - 0.25
	Itraconazole	S ≤ 0.12, SDD = 0.25, - 0.5 R ≥ 1	14.3	38.1	47.6	0.125	1	0.032 - 2
	Ketoconazole	WBP	0	0	0	0.032	0.125	0.032 - 16
Candida parapsilosis	Amphotericin B	S ≤ 1, R ≥ 1	0	0	100	0.032	0.032	0.032 - 0.5
	Caspofungin	S ≤ 2, I = 4, R ≥ 8	0	0	100	0.032	0.125	0.032 - 0.25
	Voriconazole	S ≤ 0.12, I = 0.25, - 0.5 R ≥ 1	0	0	100	0.032	0.032	0.032 - 0.032
	Fluconazole	S ≤ 2, SDD = 4, R ≥ 8	0	0	100	0.125	0.25	0.064 - 2
	Posaconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.032
	Itraconazole	S ≤ 0.12, SDD = 0.25, - 0.5 R ≥ 1	0	60	40	0.125	0.5	0.032 - 0.5
	Ketoconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.064
Candida kefyr	Amphotericin B	S ≤ 1, R ≥ 1	0	0	100	0.032	1	0.032 - 1
	Caspofungin	S ≤ 2	0	0	100	0.032	0.125	0.032 - 0.125
	Voriconazole	WBP	0	0	0	0.032	0.032	0.032-0.032
	Fluconazole	S ≤ 8, R ≥ 64	0	0	100	0.5	0.5	0.25 - 0.5
	Posaconazole	WBP	0	0		0.032	0.032	0.032 - 0.032
	Itraconazole	S ≤ 0.12, R ≥ 1	0	16.7	83.3	0.032	0.125	0.032 - 0.125
	Ketoconazole	WBP	0	0		0.032	0.032	0.032 - 0.032

[a] Resistant
[b] Intermediate
[c] Sensitive
[d] This drug doesn't have any breakpoint (without any breakpoint)

Antifungal resistance may occur due to cross-reactivity of azole antifungal agents. Such a trend was reported using FLU and ITR as prophylaxis or treatment in patients, leading to resistant species, and treatment with other azoles failed. Resistance rate to FLU varied in different studies and for *C. glabrata* it was reported as 95% (38/40), 6.2% (1/16) and 10.3% (6/58 with 52/58 susceptible dose dependent) (6, 11, 23). These rates for *C. krusei* were 95.2% (59/62) (6). *Candida krusei* is naturally resistant to antifungal drugs, especially FLU (24). In the current study, ITR resistance rate in *C. albicans* was 12.7% (34.7/273) with dose dependent susceptibility of 35.3% (96/273). This rate was reported as 15.1% (26/172), 5.4% (2/38), 28% (36/117) and 11.9% (18/167) in other studies (6, 11, 15, 25). Itraconazole resistance rates in *C. glabrata* and *C. krusei* in the present study were 77.8% (14/18) and 33.3% (3/10), and in other studies, these were 85% (34/40) and

Table 3. Sensitivity Pattern of Uncommon Candida Species Isolated From Clinical Samples in Six University Hospital in Iran

Candida	Antibiotic name	Breakpoints	%R	%I	%S	MIC50	MIC90	MIC Range
Candida famata	Amphotericin B	S ≤ 1, R ≥ 1	5	0	95	0.032	0.125	0.032 - 2
	Caspofungin	S ≤ 2	0	0	94.7	0.032	0.5	0.032 - 16
	Voriconazole	WBP	0	0	0	0.032	0.25	0.032 - 0.5
	Fluconazole	S ≤ 8, R ≥ 64	0	0	100	0.25	0.5	0.064 - 8
	Posaconazole	WBP	0	0	0	0.032	0.5	0.032 - 1
	Itraconazole	S ≤ 0.12, R ≥ 1	10	35	55	0.032	0.5	0.032 - 1
	Ketoconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.25
Candida glabrata	Amphotericin B	S ≤ 1, R ≥ 1	11.1	0	88.9	0.032	16	0.032 - 16
	Caspofungin	S ≤ 0.12, I = 0.25, R ≥ 0.5	22.2	11.1	66.7	0.032	8	0.032 - 8
	Voriconazole	ECV[a] = 0.5	0	0	0	0.032	4	0.032 - 4
	Fluconazole	SDD ≤ 32, R ≥ 64	11.1	0	88.9	0.5	64	0.064 - 64
	Posaconazole	WBP	0	0	0	0.125	8	0.032 - 8
	Itraconazole	S ≤ 0.12, SDD = 0.25, - 0.5 R ≥ 1	77.8	0	22.2	1	16	0.032 - 16
	Ketoconazole	WBP	0	0	0	0.064	4	0.032 - 4
Candida krusei	Amphotericin B	S ≤ 1, R ≥ 1	30	0	70	1	8	0.032 - 8
	Caspofungin	S ≤ 0.25, I = 0.5, R ≥ 1	33.3	0	66.7	0.125	2	0.032 - 2
	Voriconazole	S ≤ 0.5, I = 1, R ≥ 2	33.3	16.7	50	0.064	2	0.032 - 2
	Fluconazole	NR[b]	0	0	0	32	64	2 - 64
	Posaconazole	WBP	0	0	0	0.125	0.5	0.032 - 0.5
	Itraconazole	S ≤ 0.12, SDD = 0.25, - 0.5 R ≥ 1	33.3	33.3	33.3	0.125	1	0.064 - 1
	Ketoconazole	WBP	0	0	0	0.064	4	0.064 - 4
***Candida* spp[c]**	Amphotericin B	S ≤ 1, R ≥ 1	0	0	100	0.032	0.032	0.032 - 0.032
	Caspofungin	S ≤ 2	0	0	100	0.032	0.064	0.032 - 0.064
	Voriconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.032
	Fluconazole	S ≤ 8, R ≥ 64	0	0	100	0.25	4	0.125 - 4
	Posaconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.032
	Itraconazole	S ≤ 0.12, R ≥ 1	0	0	80	0.032	0.125	0.032 - 0.125
	Ketoconazole	WBP	0	0	0	0.032	0.032	0.032 - 0.032

[a] Epidemiological cut off value
[b] Naturally resistant (This species is resistant to fluconazole with every MICs)
[c] *Candida dubliniensis*, *Candida guilliermondii* and *Candida lusitaniae*

85.5% (53/62), and 50% (7/14) and 30% (6/18), respectively (6, 15). Voriconazole is a drug of choice for the treatment of filamentous fungi and it has a good activity against fluconazole-resistant *C. glabrata* strains (26). According to literature, low resistance rate of VOR for *Candida* species was reported (6, 14, 27).

Ketoconazole showed significant toxicity as a systemic drug, so it is only available as a topical drug (cream and shampoo) for the treatment of cutaneous fungal infections (26). Posaconazole and KET do not have any breakpoint mentioned in CLSI M27-S4 reference (10). Previously, highest MIC values for KET were reported in *C. krusei* (4 μg/mL) (15) and *C. albicans* (32 and 2 μg/mL) (15, 28). The difference could be explained by the sample size and study population, e.g., in immunocompromised cases, like HIV patients, the resistance rate to anti-fungal agents was high (28). In this study, highest MIC90 value for POS was observed in *C. glabrata* (2 μg/mL), as in a previous study (15). In a study from 70 medical centers around the world, the highest MIC90 value for POS was reported in *C. glabrata*

with 2 μg/mL (29).

6. Conclusion

Selection of the most appropriate drug and effective treatment for patients is critical in clinical practice. According to this study, *C. albicans* is the most prevalent etiologic agent in infected patients and caspofungin is the most effective antifungal agent. Regional data may not be applicable to other regions and may lead to failure in treatment. Knowledge about etiologic agents and their susceptibility patterns in each region is required for successful treatment of patients.

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Footnotes

Authors' Contribution: Parisa Badiee Study concept and design, Parisa Badiee, Hamid Badali, Kambiz Diba, Rasoul Mohammadi, Hossein Mirhendi, and Mohammad Javad Najafzadeh Acquisition of data, Parisa Badiee and Hadis Jafarian Analysis and interpretation of data, Parisa Badiee and Hadis Jafarian Drafting of the manuscript, Parisa Badiee, Hamid Badali, Kambiz Diba, Rasoul Mohammadi, Hossein Mirhendi and Mohammad Javad Najafzadeh Critical revision of the manuscript for important intellectual content, Parisa Badiee and Hadis Jafarian Statistical analysis, Parisa Badiee and Hadis Jafarian Administrative, technical, and material support, Parisa Badiee Study supervision.

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