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Microdilution in vitro Antifungal Susceptibility Patterns of *Candida* Species, From Mild Cutaneous to Bloodstream Infections

Elham Rezazadeh,[1] Azar Sabokbar,[1] Maryam Moazeni,[2] Mohammad Sadegh Rezai,[3] and Hamid Badali[4,*]

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, IR Iran
[2]Department of Medical Mycology and Parasitology, Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, IR Iran
[3]Infection Diseases Research Center with Focus on Nosocomial Infection, Mazandaran University of Medical Sciences, Sari, IR Iran
[4]Department of Medical Mycology and Parasitology, Infection Diseases Research Center with Focus on Nosocomial Infection, Mazandaran University of Medical Sciences, Sari, IR Iran

[*]*Corresponding author*: Hamid Badali, Department of Medical Mycology and Parasitology, Infection Diseases Research Center with Focus on Nosocomial Infection, Mazandaran University of Medical Sciences, Sari, IR Iran. Tel: +98-9128413720, E-mail: badalii@yahoo.com

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Abstract

Background: *Candida* species, as opportunistic organisms, can cause various clinical manifestations, ranging from mild cutaneous infections to systemic candidiasis in otherwise healthy individuals. Remarkably, the incidence and mortality rates of candidemia have significantly increased worldwide, even after advances in medical interventions and the development of novel antifungal drugs.

Objectives: Given the possible resistance to antifungal agents, susceptibility testing can be useful in defining the activity spectrum of antifungals and determining the appropriate treatment regime.

Materials and Methods: The in vitro susceptibilities of molecularly identified *Candida* strains (n = 150) belonging to seven species recovered from clinical specimens, including vaginal, cutaneous, sputum, bronchoalveolar lavage (BAL), and blood samples, were determined for six antifungal drugs (amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin), based on the clinical and laboratory standards institute's M27-A3 and M27-S4 documents.

Results: *Candida albicans* was the most frequently isolated species (44.66%), followed by non-albicans *Candida*, including *C. glabrata* (20%), *C. parapsilosis* (13.33%), *C. krusei* (8%), *C. tropicalis* (7.3%), *C. dubliniensis* (4%), and *C. africana* (3.33%). Posaconazole had the lowest geometric mean minimum inhibitory concentration (MIC) (0.0122 μg/ml), followed by amphotericin B (0.0217 μg/mL), voriconazole (0.1022 μg/mL), itraconazole (0.1612 μg/mL), caspofungin (0.2525 μg/mL), and fluconazole (0.4874 μg/mL) against all isolated *Candida* species. *Candida africana* and *C. parapsilosis* were significantly more susceptible to fluconazole, compared to *C. albicans* and other *Candida* species ($P < 0.001$). However, their clinical effectiveness in the treatment of *Candida* infections remains to be determined.

Conclusions: These findings highlight the importance of precise and correct species identification of clinical yeast isolates via molecular approaches, and of monitoring the antifungal susceptibility of *Candida* species recovered from clinical sources. Laboratories should consider routine MIC testing of *C. glabrata* isolates collected from sterile sites. Surveillance studies of *Candida* species and new analyses of antifungal treatment outcomes will allow more informed determinations of the value of these drugs in the antifungal armamentarium.

Keywords: Candidiasis, Antifungal Susceptibility, *Candida* Species

1. Background

Candida species, as opportunistic organisms, can cause various clinical manifestations, ranging from mild cutaneous infections to systemic candidiasis in otherwise healthy individuals. In recent decades, the incidence of candidiasis has dramatically increased, especially in human immunodeficiency virus (HIV) patients, immunocompromised hosts, intravenous drug abusers, and patients with underlying valvular heart disease, prosthetic valve implants, or prolonged use of intravenous catheters (1, 2). Even after advances in medical interventions and the development of novel antifungal drugs, the mortality rate remains high and has been reported to range between 40% and 70% in different studies.

Although *C. albicans* is an important and global cause of morbidity and mortality among patients admitted to intensive care units (ICUs) or oncology wards, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and atypical forms of *C. albicans* (i.e., *C. africana*, *C. dubliniensis*, and *C. stellatoidea*), may also be involved (3, 4). These species are closely correlated in phylogenetic terms, and a treatment regimen against one

species is generally effective against others (4). However, several reports have shown variable susceptibilities of *Candida* species to diverse antifungal agents; in addition, some species have acquired resistance to particular antifungal drugs (5, 6). The widespread use of antifungals and the prolonged duration of therapy are regarded as risk factors for the emergence of antifungal resistance in *Candida* species (7). Among recently developed triazole and echinocandin agents, posaconazole and caspofungin appear to be highly active against all *Candida* species, including those less susceptible or resistant to fluconazole and/or itraconazole (8, 9). The infecting species, site of infection, and rate of drug accessibility to the infected tissue are major factors that must be considered for the selection of an appropriate treatment.

2. Objectives

In vitro antifungal susceptibility testing is considered a powerful method for determining the drug of choice against various forms of candidiasis. In Iran, limited data is available on the susceptibility profiles of fungi against currently used antifungal agents. In the current study, we evaluated the in vitro antifungal susceptibility of six antifungal agents, i.e., amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin, against 150 *Candida* strains isolated from different clinical sources.

3. Materials and Methods

3.1. Fungal Strains

One hundred fifty clinical specimens were recovered from patients with candidiasis. The samples consisted of clinical isolates from a variety of sources, including vaginal, cutaneous, sputum, bronchoalveolar lavage (BAL), and blood specimens. The obtained strains were preliminarily identified to the species level based on microscopic and macroscopic characteristics, i.e., germ tube tests in the serum at 37°C for 2 - 3 hours in darkness, green colony color of the isolates on CHROMagar *Candida* medium (CHROMagar company, Paris, France), morphology on cornmeal agar (CMA, Difco laboratories, Detroit, MI, USA) supplemented with 1% tween 80, and carbohydrate assimilation tests using the ID 32C kit (BioMerieux, France) (10). Identified strains were deposited to the reference culture collection of the invasive fungi research center (IFRC), Sari, Iran, and subsequently their identities were reconfirmed by molecular analysis. The study protocol was reviewed and approved by the ethics committee of Mazandaran University of Medical Sciences (nr. 799) and Islamic Azad University, Karaj, Iran (ethics code: 14291).

3.2. Molecular Investigations

DNA sequencing was performed in order to re-confirm the identity of the isolates, which were recognized as *Candida* species based on conventional methods. Briefly, DNA was extracted from 3-day-old cultures with an UltraClean® Microbial DNA Isolation Kit (Mobio, Carlsbad, CA, USA) according to the manufacturer's protocol, then stored at -20°C prior to use. The internal transcribed spacer (ITS rDNA) region was amplified and sequenced using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), which have been described (9, 11).

Briefly, the amplification of ITS rDNA was performed with a cycle of 5 min at 94°C for primary denaturation, followed by 35 cycles at 94°C (30 seconds), 52°C (30 seconds), and 72°C (80 seconds), with a final 7 min extension step at 72°C. PCR products were run in 1.2% agarose gels and visualized with UV after ethidium bromide staining, then were purified using GFX PCR DNA (GE Healthcare, Ltd., Buckinghamshire, UK). Amplicons were then subjected to direct sequencing using ABI prism BigDye® terminator cycle sequencing kit (applied biosystems, foster city, CA, USA) and analyzed on an ABI Prism 3730XL Sequencer. The obtained sequence data were adjusted using SeqMan of the Lasergene software (DNAStar Inc., Madison, WI, USA) and compared with the GenBank database (http://blast.ncbi.nlm.nih.gov).

3.3. Antifungal Susceptibility Testing

In vitro antifungal susceptibility tests using minimum inhibitory concentrations (MICs) were assayed for the identified *Candida* species of *C. albicans* (n = 67), *C. glabrata* (n = 30), *C. parapsilosis* (n = 20), *C. krusei* (n = 12), *C. tropicalis* (n = 11), *C. dubliniensis* (n = 6), and *C. africana* (n = 4) according to the recommendations in the clinical and laboratory standards institute (CLSI) M27-A3 and M27-S4 documents (12, 13). Amphotericin B (Sigma, St. Louis, MO, USA), fluconazole (Pfizer, Groton, CT, USA), itraconazole (Janssen research foundation, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Schering-Plough, Kenilworth, USA), and caspofungin (Merck, Whitehouse Station, NJ, USA) were obtained from their respective manufacturers as reagent-grade powders for preparation of the CLSI microdilution trays.

The antifungal agents were diluted in the standard RPMI-1640 medium (Sigma chemical Co., USA) buffered to pH 7.0 with 0.165 M-morpholinepropanesulfonic acid (MOPS) (Sigma), with L-glutamine without bicarbonate, to yield two times their concentrations. They were dispensed into 96-well microdilution trays at a final concentration of 0.016 - 16 μg/mL for amphotericin B, itraconazole, voriconazole, and posaconazole, 0.063 - 64 μg/mL for fluconazole,

and 0.008 - 8 μg/mL for caspofungin. The plates were stored at -70°C until they were used. Briefly, all isolates were grown on potato dextrose agar (PDA, Difco, Leeuwarden, the Netherlands) plates at 35°C for up to three days, with the inoculum suspensions being prepared by lightly scraping the surface of mature colonies with a loop and the resulting material being suspended in sterile saline solution.

The homogeneous conidial suspensions were then transferred to sterile tubes and the supernatants were adjusted spectrophotometrically at a wavelength of 530 nm, to an optical density (OD) that ranged from 0.12 to 0.11 (2.5 - 5 $\times 10^6$ CFU/mL). Microdilution plates were incubated at 35°C and examined visually after 24 and 48 hours to determine the MIC values. The MIC endpoints were determined with the aid of a reading mirror and were defined as the lowest concentration of drug that prevents any recognizable growth (i.e., exerts 100% inhibition for amphotericin B) or significant (50%) growth diminution levels (for all other agents) compared with the growth of a drug-free control. The *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) strains were chosen as quality controls, and analysis of these strains was performed with every new batch of MIC plates.

4. Results

Table 1 summarizes the results of MIC range, geometric mean MIC, MIC50, and MIC90; however, MIC90 was not measured when fewer than nine isolates were available. The MIC values of the tested antifungal agents revealed high susceptibility in all *C. albicans* complex isolates. The results showed the widest range and highest MICs for fluconazole (0.016–>16 μg/ml) and itraconazole (0.016 - 16 μg/mL), respectively. The uniform patterns of low MIC ranges in all *Candida* species were, in increasing order: posaconazole (0.008 - 0.063 μg/mL), amphotericin B (0.008 - 0.25 μg/mL), caspofungin (0.016 - 4 μg/mL), voriconazole (0.016 - 8 μg/mL), itraconazole (0.016 - 16 μg/mL), and fluconazole (0.016 -> 16 μg/mL).

Basically, in terms of MIC50, the values for posaconazole, amphotericin B, voriconazole, and itraconazole were low for all tested *Candida* strains (Table 1). Based on these findings, the variation in MIC90 values between *C. albicans* strains was not more than one dilution step. Geometric mean MICs of caspofungin were >4-$\log_2$-dilution and >2-$\log_2$-dilution less active than posaconazole and amphotericin, respectively. Posaconazole was the most active agent, followed by voriconazole, itraconazole, amphotericin B, and caspofungin. Based on the revised interpretative guidelines for MICs of *Candida* species, for *C. krusei* and *C. glabrata*, fluconazole ($\geq$ 8 and $\geq$ 64 μg/mL) and caspofungin ($\geq$ 1 and $\geq$ 0.5 μg/mL), respectively, are used to identify resistance. Therefore, the results showed that in terms of MIC90, all *C. krusei* and *C. glabrata* isolates recovered from different sources were resistant to fluconazole and caspofungin, but not to itraconazole or amphotericin B.

In contrast, all *C. albicans* complex species (*C. albicans*, *C. africana*, and *C. dubliniensis*) and *C. parapsilosis* strains were highly susceptible to fluconazole. Remarkably, *C. africana* and *C. parapsilosis* were more susceptible to fluconazole, compared to *C. albicans* and other non-albicans *Candida* species ($P < 0.001$). For all *C. africana* strains, the GM MIC of fluconazole was 2-$\log_2$-dilution steps less active than posaconazole. Also, amphotericin B was 2-$\log_2$-dilution steps and 3-$\log_2$-dilution steps less active than the azole and echinocandin agents, respectively. The overall frequency of fluconazole resistance in the evaluated data set was 20.3%. The present results revealed a statistically significant difference in the susceptibility of *C. albicans* and non-albicans *Candida* to fluconazole and itraconazole ($P < 0.05$).

5. Discussion

Candidiasis is one of the most severe fungal infections in immunocompromised individuals (14). The most common risk factors for candidiasis include HIV, prolonged antibiotic use, central venous catheterization, parenteral nutrition, ICU administration and intravenous drug abuse (1, 4). The susceptibility analysis in the present study is comparable with reports from different continents with slight differences, which are dependent on the underlying diseases and *Candida* species involved in the infection. Comparison of susceptibility to antifungal agents is of limited function in evaluating nosocomial *Candida* infections (14-16).

In the current study, we present the largest published dataset for clinically important *Candida* species. *Candida albicans* (40.1%) was recognized as the most common agent of candidiasis, followed by non-albicans species. However, it should be noted that infections caused by *C. dubliniensis*, *C. africana*, *C. tropicalis*, and *C. krusei* are quite rare; in fact, only a few cases have been detected and successfully treated via antifungal therapies. Although the present results were in concordance with previous research (4, 9, 17), the total percentage of non-albicans *Candida* (51%) was higher than the previous reports (7, 9). Consistent with the literature (17), our findings demonstrated a remarkable increase in the recovery of non-albicans *Candida* from clinical specimens.

According to a study by Sobel et al., *C. albicans* was the most frequently isolated species, followed by *C. glabrata*, *C.*

tropicalis, *C. parapsilosis*, and *C. krusei* (18). Similarly, in a study by Badiee et al., the most frequently isolated species from patients were *C. albicans* (50%), *C. glabrata* (21.4%), *C. dubliniensis* (13.3%), *C. krusei* (9.8%), *C. kefyr* (3.1%), *C. parapsilosis* (1.6%), and *C. tropicalis* (0.8%) (16). On the other hand, in a study by Panizo et al., diversity in *Candida* species was dependent on types of health care systems, clinical specimen, and the geographical region where the study was performed (19). According to a study by Pfaller et al., the prevalence of *C. glabrata* (19.2%) among patients with multiple positive vaginal cultures was higher than that reported among bloodstream isolates (8% - 18%) (20).

In some European and South American countries, *C. parapsilosis* is the second or even the first most common etiological agent of candidemia; this species is especially important in ICUs (21, 22). The susceptibility analyses of the tested antifungal drugs in the present study are comparable with reports from different continents with slight differences, which are dependent on the underlying diseases and *Candida* species involved in the infection. Comparison of susceptibility to antifungal agents is of limited function in evaluating nosocomial *Candida* infections (23).

Based on the susceptibility testing in the present study, posaconazole showed the lowest MICs for all *Candida* species. As expected, higher MIC values for azoles were reported among non-albicans *Candida* species, and fluconazole exhibited the highest MIC90 among the tested azoles (8 μg/mL). However, in the current study, the susceptibility of all *C. albicans* isolates to fluconazole was consistent with previous research (7, 9). The MIC ranges of amphotericin B and posaconazole for all *Candida* species were 0.008 - 0.25 μg/mL and 0.008 - 0.063 μg/mL, respectively. The results showed that all species complexes of *C. albicans* and *C. parapsilosis* strains were susceptible to fluconazole; however, fluconazole resistance was mainly observed in *C. krusei* (0.016 - 64 μg/mL) and partially in *C. glabrata* ($0.016 \geq 64$ μg/mL). In contrast, based on various studies, *C. glabrata*, followed by *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and atypical *Candida* species showed resistance to itraconazole rather than to fluconazole.

These results were slightly different from those reported in the United States, Europe, and Latin America (19). In fact, the prevalence of candidiasis caused by non-albicans *Candida* species (such as *C. glabrata*, which is intrinsically resistant to fluconazole) is on the rise among immunocompromised patients (24). Similarly, in previous studies, among the species complexes of *C. albicans*, *C. africana*, *C. dubliniensis*, and *C. stellatoidea* appeared to be highly susceptible to the tested antifungals. Consequently, we conclude that most *Candida* infections seem to be caused by *C. albicans*. Also, the observed azole susceptibility supports the continued use of azole agents for the empirical therapy of candidiasis. Based on recent findings reported by international surveillance studies, no increase has been detected in triazole resistance. Understanding the correlation between in vitro resistance and outcomes can be a significant challenge in medically complex patients. Therefore, further research is highly recommended to enable a thorough analysis of epidemiological changes, which highlight an increase in non-albicans *Candida* isolates over *C. albicans* (5).

In conclusion, these findings highlight the importance of precise and correct species identification of clinical yeast isolates via molecular approaches and the monitoring of antifungal susceptibility of *Candida* species recovered from clinical sources. Surveillance studies of *Candida* species and new analyses of antifungal treatment outcomes will allow more informed determinations of the value of these drugs in the antifungal armamentarium.

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Footnotes

Authors' Contribution: Study concept and design, Hamid Badali and Azar Sabokbar; acquisition of data, Elham Rezazadeh; analysis and interpretation of data, Hamid Badali and Elham Rezazadeh; drafting of the manuscript, Hamid Badali, Mohammad Sadegh Rezai, and Elham Rezazadeh; critical revision of the manuscript, Hamid Badali, Maryam Moazeni, and Azar Sabokbar; study supervision and co-supervision, Hamid Badali, Azar Sabokbar, and Maryam Moazeni, respectively.

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Table 1. In vitro Antifungal Susceptibilities of Six Antifungal Drugs Against 150 *Candida* Species Isolated From Mild Cutaneous to Bloodstream Infections

Antifungal Agents	MIC, μg/mL			
	MIC Range	MIC_{50}	MIC_{90}	G Mean
All strains of *Candida* species (n = 150)				
AmB	0.008 - 0.25	0.016	0.125	0.0217
FLC	0.016 - >16	0.5	8	0.4874
ITC	0.016 - 16	0.125	2	0.1612
VRC	0.016 - 8	0. 25	2	0.1022
POS	0.008 - 0.063	0.008	0.016	0.0122
CAS	0.016 - 4	0.25	2	0.2525
***Candida albicans* (n = 67; 44.66%)**				
AmB	0.008 - 0.125	0.016	0.25	0.0205
FLC	0.016 - 4	0.5	1	0.5192
ITC	0.016 - 0.25	0.016	0.25	0.0254
VRC	0.016 - 0.25	0.016	0.125	0.0215
POS	0.016 - 0.063	0.016	0.016	0.0110
CAS	0.016 - 2	0.016	0.0.5	0.0919
***Candida glabrata* (n = 30; 20%)**				
AmB	0.016 - 0.25	0.016	0.25	0.0356
FLC	0.016 - > 64	8	64	4.0036
ITC	0.25	4	8	2.5198
VRC	0.25	1	2	1.5642
POS	0.063 - 2	0.25	0.5	0.2721
CAS	0.25	0.5	1	0.4719
***Candida parapsilosis* (n=20; 13.33%)**				
AmB	0.016 - 0.5	0.016	0.638	0.0212
FLC	0.016 - 2	0.031	2	0.1099
ITC	0.25 - 4	2	4	1.0256
VRC	0.25 - 0.5	0.5	0.5	0.0212
POS	0.016 - 0.25	0.031	0.125	0.03732
CAS	0.125 - 2	1	2	0.9330
***Candida krusei* (n = 12; 8%)**				
AmB	0.125 - 4	0.25	0.5	0.0264
FLC	0.016 - 64	8	64	4.1036
ITC	0.016 - 2	0.5	1	0.8542
VRC	0.125 - 2	0.5	1	0.4526
POS	0.125 - 0.063	0.125	0.25	0.1012
CAS	0.016 - 4	0.5	2	0.9325
***Candida tropicalis* (n = 11; 7.33%)**				
AmB	0.016 - 1	0.13	0.25	0.0354
FLC	0.125 - 16	0.5	1	0.4874

ITC	0.016 - 8	0.13	0.5	0.0213
VRC	0.016 - 1	0.125	0.25	0.0125
POS	0.016 - 0.5	0.031	0.031	0.1250
CAS	0.031 - 2	0.5	0.5	0.4719
Candida dubliniensis **(n = 6; 4%)**				
AmB	0.002 - 0.016	0.016	ND	0.0081
FLC	0.016 - 0.5	0.125	ND	0.0945
ITC	0.125 - 4	1	ND	0.7071
VRC	0.125 - 1	0.5	ND	0.4582
POS	0.031 - 0.125	0.031	ND	0.0310
CAS	0.25 - 2	0.75	ND	0.7071
Candida africana **(n = 4; 2.66%)**				
AmB	0.016 - 0.25	0.25	ND	0.3149
FLC	0.031 - 0.25	0.25	ND	0.3149
ITC	0.016 - 0.031	0.016	ND	0.0165
VRC	0.016 - 0.031	0.031	ND	0.1256
POS	0.008 - 0.016	0.008	ND	0.0101
CAS	0.016 - 0.031	0.031	ND	0.1256

Abbreviations: AmB, amphotericin B; CAS, caspofungin; FLC, fluconazole; GM, geometric mean; ITC, itraconazole; MIC50, concentration at which 50% of the isolates were inhibited; MIC90, concentration at which 90% of the isolates were inhibited; POS, posaconazole; VRC, voriconazole.