

## Original Article

***In vitro* activity of five antifungal agents against *Candida albicans* isolates, Sari, Iran**Shokohi T<sup>1,2</sup>, Badali H<sup>1,2</sup>, Amirrajab N<sup>3</sup>, Ataollahi MR<sup>4</sup>, Kouhpayeh SA<sup>5</sup>, Afsarian MH<sup>4\*</sup><sup>1</sup>Invasive Fungal Research Center, Mazandaran University of Medical Sciences, Sari, Iran<sup>2</sup>Department of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran<sup>3</sup>Department of Medical Laboratory Sciences, School of Paramedicine/Infectious & Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran<sup>4</sup>Department of Medical Microbiology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran<sup>5</sup>Department of Pharmacology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran**\*Corresponding author: Mohammad Hosein Afsarian**, Department of Medical Microbiology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.

Email: afsariyan@gmail.com

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**Abstract****Background and Purpose:** *Candida albicans* is the most common causative agent of candidiasis. Candidiasis management is dependent on the immune status of the host, severity of disease, and the choice of antifungal drug. Antifungals, specifically triazoles, are widely administered for the treatment of invasive fungal infections. Herein, we aimed to evaluate the *in vitro* susceptibility of *C. albicans* isolates to fluconazole (FLZ), itraconazole (ITZ), voriconazole (VRZ), amphotericin B (AMB), and Caspofungin (CAS).**Materials and Methods:** A total of 44 clinical strains of *C. albicans* were collected from 36 patients admitted to four hospitals in Mazandaran Province, Iran. The *in vitro* antifungal susceptibility testing was performed based on the Clinical and Laboratory Standards Institute methods.**Results:** Generally, 34 isolates were susceptible to all the five antifungal drugs, while four isolates were susceptible or susceptible dose-dependent (SDD) and six isolates were SDD or resistant to these antifungals. The lowest minimum inhibitory concentration (MIC; 0.016 µg/ml) belonged to AMB and the highest MIC was for FLZ (16 µg/ml). The lowest MIC<sub>50</sub> (0.063 µg/ml) was related to ITZ and the lowest MIC<sub>90</sub> (0.25 µg/ml) pertained to CAS; in addition, the highest MIC<sub>50</sub> (1 µg/ml) and MIC<sub>90</sub> (4 µg/ml) were for FLZ. Four of the isolates showed resistance to both FLZ and VRZ, separately, and five isolates were resistant to ITZ. Caspofungin showed potent activity against more than 95% of the *C. albicans* isolates.**Conclusion:** Overall, we reported 9.1% resistance to FLZ and VRZ, 11.3% resistance to ITZ and AMB, and 4.6% resistance to caspofungin. Our finding is in agreement with previous observations proposing that *C. albicans* isolates develop resistance to some antifungal drugs such as FLZ since they are widely used as prophylaxis.**Keywords:** Antifungal agents, *Candida albicans*, *In vitro* susceptibility testing**► How to cite this paper:**Shokohi T, Badali H, Amirrajab N, Ataollahi MR, Kouhpayeh SA, Afsarian MH. *In vitro* activity of five antifungal agents against *Candida albicans* isolates, Sari, Iran. Curr Med Mycol. 2016; 2(2): 34-39. DOI: 10.18869/acadpub.cmm.2.2.8**Introduction**

**C***andida* species remain the predominant cause of invasive fungal infections; among them, *Candida albicans* is the most prevalent *Candida* species in clinical samples [1-3], which are the fourth leading cause of nosocomial bloodstream infections in the United States [4, 5]. Currently, systemic candidiasis is uncommon, but its incidence is on a growing trend, particularly among severely immunocompromised individuals [6-11]. Management of candidiasis depends on the immune status of the host, severity of the disease, and the choice of antifungal drug [12].

Voriconazole (VRZ) and caspofungin (CAS), as novel drugs, are used for the treat-

ment of invasive fungal infections. In particular, VRZ shows broad-spectrum activity against clinically relevant yeasts. Treatment recommendations are based on numerous studies, however, routine prophylactic and therapeutic regimen of fluconazole (FLZ) in admitted and immunocompromised patients for management of candidiasis can lead to a shift toward resistant strains [3, 6-8, 13]. Several studies have reported increased FLZ resistance rates in *C. albicans* isolates [14], but new antifungal agents with a better activity may help improve the management of these infections [6, 7, 15-17].

In the current investigation, we aimed to evaluate the *in vitro* susceptibility of a collection of *C. albicans* to amphotericin B (AMB), fluconazole (FLZ), voriconazole (VRZ), itraconazole (ITZ) and caspofungin (CAS) based on microdilution broth.

## Materials and Methods

A total of 44 clinical isolates of *C. albicans* were collected from 36 patients admitted to four hospitals in Mazandaran Province, Iran, due to burn injury, pulmonary infection, solid tumor, and cancer. The isolates were obtained from various sources including blood (n=7), burn wound (n=19), bronchoalveolar lavage (BAL) (n=7), oropharynx (n=7), urine (n=3), and sputum (n=1) [10, 11]. The study protocol was approved by the Ethics Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.91-32). Correct identification of isolates was previously performed by sequence analysis of the internal transcribed spacer (ITS) regions of rDNA followed by seven housekeeping genes (i.e., *AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, and *ZWP1b*) [10, 11].

Briefly, fresh colonies from Sabouraud glucose agar (SGA, Difco) were selected for gDNA extraction, and sequencing was performed on an ABI 3730xl automated sequencer (Applied Biosystems, US). Sequence data obtained in this study were adjusted using the SeqMan of Lasergene software (DNASTar Inc., US) and were compared through GenBank database and the local database at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands.

Minimum inhibitory concentration (MIC) was determined according to the recommendations proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4 documents [18, 19]. AMB (Sigma, St. Louis, MO, USA), FLZ (Pfizer, Groton, CT, USA), ITZ (Janssen Research Foundation, Beerse, Belgium), VRZ (Pfizer), and CAS (Merck, Whitehouse Station, NJ, USA) were obtained as reagent-grade powders from the respective manufacturers for preparation of the microdilution trays based on CLSI recommendations.

The antifungal agents were diluted in the standard RPMI-1640 medium (Sigma Chemical Co. Germany) buffered to pH 7.0 with 0.165 M-morpholinepropanesulfonic acid (MOPS) (Sigma, Germany) with L-glutamine without bicarbonate to yield two times their

concentrations. The buffer was dispensed into 96-well microdilution trays at a final concentration of 0.016–16 µg/ml for AMB, ITZ, and VRZ, 0.063–64 µg/ml for FLZ, and 0.008–8 µg/ml for CAS. Plates were stored at -70°C until they were used.

Briefly, homogeneous conidial suspensions were spectrophotometrically measured at the 530 nm wavelength and a percent transmission within the range of 75–77%. Therefore, the final densities of the stock inoculum suspensions of the tested isolates ranged between  $2.5 \times 10^3$  and  $5 \times 10^3$  colony forming units/ml, as determined by quantitative colony count on Sabouraud glucose agar (SGA, Difco). The 96-well microplates were incubated at 35°C and examined visually after 24 and 48 h to determine MIC values.

The MIC endpoints were determined using 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 level (all other agents) compared with the growth of a drug-free control. We used the recently revised CLSI clinical breakpoint (CBP) values to identify the *C. albicans* strains [19]. Caspofungin MIC values of  $\leq 0.25$  and  $\geq 1$  µg/ml were categorized as susceptible and resistant, respectively; fluconazole MIC results of  $\leq 2$  and  $\geq 8$  µg/ml were defined as susceptible and resistant, respectively, and CLSI susceptible and resistant breakpoints for VRZ and ITZ were  $\leq 0.12$  and  $\geq 1$  µg/ml, respectively.

CBPs are not established for AMB in the CLSI M27-S4 [19]. Isolates with MIC  $\geq 2$  µg/ml for AMB were considered resistant. Isolates were classified based on both the previously and the recently revised CLSI clinical breakpoints. Quality control was performed as recommended in CLSI document M27-A3 [18] using *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains. All the tests were performed in duplicate, and differences among mean values were determined by Student's t-test, using SPSS version 7.0. P-value less than 0.05 was considered statistically significant.

## Results

Table 1 summarizes the *in vitro* susceptibility patterns of 44 clinical isolates of *C. albicans*, including MIC<sub>50</sub>, MIC<sub>90</sub>, the geometric mean MIC values, and the range of MIC values of the five antifungal agents (Table 1).

**Table 1.** *In vitro* susceptibility testing of 44 clinical isolates of *C. albicans* to five antifungal agents (minimum inhibitory concentration range, geometric (G) mean, MIC<sub>50</sub>, and MIC<sub>90</sub> values are expressed in µg/ml)

Antifungal	MIC <sup>1</sup> range	MIC <sub>50</sub>	MIC <sub>90</sub>	G mean	R <sup>2</sup> (N/%)	SDD <sup>3</sup> (N/%)	I <sup>4</sup> (N/%)
Fluconazole	0.25-16	1	4	1.099	4 (9.1)	3 (6.8)	-
Itraconazole	0.031-4	0.063	1	0.125	5 (11.3)	5 (11.3)	-
Voriconazole	0.031-1	0.125	0.5	0.133	4 (9.1)	5 (11.3)	-
Amphotericin B	0.016-4	0.125	1	0.129	5 (11.3)	-	-
Caspofungin	0.031-1	0.125	0.25	0.156	2 (4.6)	-	3 (6.8)

MIC<sup>1</sup>: Minimum Inhibitor Concentration, R<sup>2</sup>: Resistant, SDD<sup>3</sup>: Susceptible Dose Dependent, I<sup>4</sup>: Intermediate

The lowest MIC (0.016 µg/ml) pertained to AMB, and MIC ranges for VRZ and CAS were the narrowest (ranging between 0.031 µg/ml and 1 µg/ml). We found the widest range and the highest MIC for FLZ (0.25-16 µg/ml). The lowest MIC<sub>50</sub> (0.063 µg/ml) was related to ITZ and the highest MIC<sub>50</sub> (1 µg/ml) belonged to FLZ; in addition, the lowest MIC<sub>90</sub> (0.25 µg/ml) was related to CAS and the highest MIC<sub>90</sub> (4 µg/ml) to FLZ. ITZ and VRZ with MIC<sub>50</sub> of 0.063 µg/ml and 0.125 µg/ml, respectively, and MIC<sub>90</sub> of 1 µg/ml and 0.5 µg/ml, respectively, were more active than FLZ with MIC<sub>50</sub> of 1 µg/ml and MIC<sub>90</sub> of 4 µg/ml (Table 1).

Generally, 34 isolates were significantly susceptible to the five antifungal drugs, while 10 isolates showed susceptibility, susceptible dose-dependent (SDD) or resistance to five antifungal drugs ( $P < 0.0001$ ). Out of these 10 isolates, three were collected from burn wound, three from BAL, two from oropharynx, one from urine, and one from blood culture [10, 11]. Four (9.1%) isolates were resistant to FLZ (MIC=8-16), four (9.1%) isolates to VRZ (MIC=1),

five (11.3%) isolates to ITZ (MIC $\geq$ 1µg/ml), and five (11.3%) isolates to AMB (MIC $\geq$ 2µg/ml; Tables 1 and 2). However, CAS showed potent activity against more than 95% of the *C. albicans* isolates, only two isolates were resistant to CAS (Table 2).

Four isolates (17, 23, 46, and 48) were SDD or resistant to the five antifungal drugs. The isolate of 17 from patient 12 (P12) with burn wound was resistant to FLZ, AMB, and CAS and was SDD to ITZ and VRZ. Isolate 46 from P28 with esophageal cancer was resistant to VRZ, ITZ, and AMB, intermediate to CAS, and SDD to FLZ; moreover, isolate 48 from P29 with esophageal cancer was resistant to FLZ, ITZ, and AMB, intermediate to CAS, and SDD to VRZ. From a total of 44 isolates, only isolate 23 from BAL sample of P24 with pulmonary infection was resistant to all the five antifungal drugs (Table 2). The isolate 6 of urine sample from P3 was resistant to ITZ and VRZ and was SDD to FLZ, but isolate 5 from blood sample of the same patient showed resistance to FLZ, ITZ, VRZ, and AMB (Table 2) [10, 11].

**Table 2.** The characterization of 10 isolates showing susceptible dose-dependent, intermediate, or resistance to five antifungal drugs

Isolate No	Patient number	Site of isolation	Fluconazole	Itraconazole	Voriconazole	Amphotericin B	Caspofungin
5	P3	Blood	R <sup>1</sup>	R	R	R	S <sup>2</sup>
6	P3	Urine	SDD <sup>3</sup>	R	R	S	S
17	P12	Chest-burn wound	R	SDD	SDD	R	R
21	P14	Hand-burn wound	SDD	SDD	SDD	S	S
23	P24	Bronchoalveolar lavage (BAL)	R	R	R	R	R
24	P25	BAL	S	SDD	SDD	S	S
25	P26	BAL	S	SDD	SDD	S	S
40	P22	Abdomen-burn wound	S	SDD	S	S	I <sup>4</sup>
46	P28	Tongue	SDD	R	R	R	I
48	P29	Mouth	R	R	SDD	R	I

R<sup>1</sup>: Resistant, S<sup>2</sup>: Susceptible, SDD<sup>3</sup>: Susceptible Dose Dependent, I<sup>4</sup>: Intermediate

## Discussion

Candidiasis is a major cause of morbidity and mortality in patients with leukemia and solid organ transplantation and those admitted to Intensive Care Units (ICUs), and *C. albicans* is the most prevalent *Candida* species in clinical samples [2, 20]. Increasing use of antifungal agents in prophylactic regimen has resulted in the development of azole resistance in *Candida* species [6].

In this study, based on CLSI guidelines, *in vitro* antifungal susceptibility testing was performed, in which six isolates showed resistance to antifungal drugs. Five patients (P2, P3, P7, P13, and P17) were sampled from different body sites [10]. Four strains isolated from four patients (P2, P7, P13 and P17) admitted to Burn Intensive Care Unit (BICU) [10] were susceptible to the five antifungal drugs at the same time. While from three strains isolated from patient 3 (P3) [10], the strain isolated from blood was resistant to three azole drugs, but was susceptible to CAS, which indicates that CAS could be a good choice for the treatment of candidemia [16, 21, 22]. Moreover, a strain isolated from urine was resistant to ITZ and VRZ and SDD to FLZ (Table 2), but the strain isolated from burn wound in the abdomen was susceptible to all the drugs.

In recent years, there have been several studies conducted on *C. albicans* using *in vitro* antifungal susceptibility testing, in which many *C. albicans* isolates displayed susceptibility to antifungal drugs [6, 23-26]. In this study, 13.6% of the isolates showed resistance to five antifungal drugs.

Previous studies comparing *in vitro* antifungal activity against *C. albicans* in Iran have reported different results [27-29]. For instance, *in vitro* susceptibility test performed for *C. albicans* isolates by Katiraei et al. [27] showed 25.7% resistance to FLZ and 100% susceptibility to CAS. Badiie and Alborzi [28] reported 10.3% resistance to FLZ, 8.5% resistance to ITZ, and 100% susceptibility to AMB, CAS, and VRZ. In addition, Shokohi et al. [29], reported 2.6% resistance to FLZ and AMB, 5.4% resistance to ITZ, and 100% susceptibility to CAS. However, in a study by Gross et al. conducted in Costa Rica [30], 100% of *C. albicans* isolates were susceptible to FLZ and ITZ.

Castanheira et al. [26] reported 100% susceptibility to AMB and CAS and 99.7%, 99.6%, and 99.1% susceptibility to VRZ, FLZ, and ITZ, respectively. Elfeky et al. [31], using disk

diffusion method, demonstrated that 10.5% of *C. albicans* isolates were resistant to FLZ and VRZ, but 100% of the isolates were susceptible to AMB. Fothergill et al. [25] revealed that FLZ resistance was increased in *C. albicans* from 2.1% to 5.7% and that VRZ resistance was 4.5%. However, we reported 9.1% resistance to FLZ and VRZ, 11.3% resistance to ITZ and AMB, and 4.6% resistance to CAS (Table 1), which is in line with findings of previous studies. It seems that widespread use of fluconazole and itraconazole for treatment or prophylaxis in Iran may be the cause of the high azole resistance rates.

Although triazoles initially appear to be highly effective, the increase of resistance to them has been reported, and many studies have substantiated that azole resistance to *Candida* isolates has been associated with widespread use of prophylaxis such as FLZ [2, 12, 29, 32, 33]. So, Ellis D. [34] showed that in some strains of *C. albicans* isolated from the patients treated with AMB, may acquire resistance during treatment.

## Conclusion

Overall, we reported 9.1% resistance to FLZ and VRZ, 11.3% resistance to ITZ and AMB, and 4.6% resistance to caspofungin. Our finding is in agreement with previous observations that resistance to antifungal drugs in *C. albicans* isolates is on a growing trend because some antifungal drugs such as FLZ are widely administered as prophylaxis [16, 21, 22, 35, 36].

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## Authors' contributions

T.S., H.B., and M.H.A. designed and managed the study and contributed to data analysis and interpretation; M.H.A wrote the main manuscript; T.S., H.B., and M.H.A revised the draft of manuscript; N.A., M.R.A., and S.A.K. set up the test and managed the research. All the authors reviewed the manuscript.

## Conflicts of interest

None declared.

## Financial disclosure

No financial interests related to the material of this manuscript have been declared.

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