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In Vitro Activity of Caspofungin Against Fluconazole-Resistant *Candida* Species Isolated From Clinical Samples in Iran

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Background: *Candida* spp. is the most common organisms involved in fungal infections in the high risk patients. It causes the greatest number of invasive candidiasis. Fluconazole is effective in treating mucosal candidiasis. However, resistance to fluconazole and other azoles antifungal drugs is an important clinical problem to treat candidiasis. Caspofungin is more effective against *Candida* species such as some azoles-resistant isolates.

Objectives: The current study aimed to investigate the susceptibilities of clinical fluconazole-resistant and fluconazole - susceptible dose-dependent *Candida* species to caspofungin.

Materials and Methods: In the Minimum Inhibitory Concentration (MIC) test, 207 *Candida* species and other yeasts isolated from Iranian patients (each isolated from a high-risk patient) were evaluated. The yeasts were differentiated by standard mycological methods, CHROM agar *Candida*, and verified by API20C.AUX. *In vitro* susceptibilities were determined using Broth Micro Dilution (BMD) method described in the Clinical Laboratory Standards Institute M27-A3. MICs were noted after 24 and 48 hours of incubation.

Results: The most frequently isolated species were *Candida albicans* (52.2%), *C. glabrata* (24.6%), followed by *C. tropicalis* (7.7%) and *C. krusei* (3.4%). MICs of caspofungin against 87% of *C. albicans* and 90% of *C. glabrata* and *C. tropicalis* isolates were 2 μg/mL and for *C. krusei* were 4 μg/mL, respectively. The results revealed that only 20 out of 207 isolates (9.7%) were non-sensitive to caspofungin. Caspofungin non-susceptible isolates were isolated from the patients with cancer, diabetes and AIDS; and not in the species isolated from patients with other underlying diseases.

Conclusions: Caspofungin appears more effective *in vitro* against Iranian fluconazole-resistant *Candida* isolates and some other yeasts.

Keywords: Fluconazole; Caspofungin; *Candida albicans*

1. Background

The most commonly used classes of antifungal agents to treat *Candida* infections are the azoles, polyenes, and echinocandins (1). Many factors including the excessive use of broad-spectrum antimicrobial agents, aggressive anticancer therapy and the AIDS epidemic increase the incidence of candidiasis (2, 3). The most common organisms involved in fungal infections in the high risk patients is *Candida* spp. that cause the greatest number of invasive candidiasis (4). Caspofungin is an echinocandins antifungal exhibiting significant *in vitro* activity against the *Candida* spp. (5). Fluconazole is effective in treating mucosal candidiasis. However, resistance to fluconazole and other azoles antifungal drugs is an important clinical problem to treat candidiasis (6). Caspofungin is more effective against *Candida* species, including some azoles-resistant isolates (6, 7). Caspofungin has excellent antifungal activity against many non-albicans *Candida* species, particularly *Candida glabrata* isolates expressing resistant to fluconazole (8-12).

Antifungal drug resistance is rapidly changing a major problem; especially with the immunocompromised patients (13, 14). Considering the increased fluconazole-resistance *Candida* spp. isolates the need arises for antifungal susceptibility testing (14, 15). In Iran, there is not enough information about the effect of caspofungin against fluconazole-resistance *Candida* spp. isolates in high-risk patients including the ones with diabetes, immunodeficiency, organs and bone marrow transplantations, cancers, immunosuppressive drugs users etc.

2. Objectives

The current study aimed to investigate the susceptibilities of clinical fluconazole-resistant and fluconazole-susceptible dose-dependent *Candida* species to caspofungin.

3. Materials and Methods

The study protocol conforms to the ethical guidelines

of the 1975 Helsinki Declaration as reflected in a priori approval by the Human Research Committee Institution and informed consent letter was obtained from each patient participated in the study (2).

3.1. Organisms

Two-hundred and seven *Candida* species including *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondi*, *C. kefyr* etc. isolated from high-risk patients including the ones with HIV-positive, cancer, diabetes and etc. and identified by standard methods (cornmeal for blastoconidia, germ-tube formation, pseudohyphae, and true hyphae and growth on Chrome *Candida* agar) and the API 20 C system (16) were examined by fluconazole in pretest; In addition, 118 ATCC 22019 and *C. krusei* ATCC 6285 which are standard species of *C. parapsilosis* included in each run of susceptibility tests for quality control.

3.2. Antifungal Drugs

To prepare caspofungin stocks, caspofungin (Merck and Co., Inc., NJ, USA) by broth micro dilution assay was used, according to the Clinical and Laboratory Standard Institute (CLSI) (16).

3.3. Preparing Caspofungin Dilution

Caspofungin stock solution, according to NCCLS micro dilution method (17), was prepared by adding 0.0256 g caspofungin to a falcon tube including 10 mL distilled water and then the tube was incubated in -70°C for further applications. RPMI 1640 (sigma Aldrich) in association with L-glutamine (without sodium bicarbonate) and buffered (pH = 7.0) with MOPS was used as susceptibility test and serial two-fold dilutions.

3.4. Culturing the Isolates

Initially, all isolates including 108 (52.2%) *C. albicans*, 51 (24.6%) *C. glabrata*, 16 (7.7%) *C. tropicalis*, 7 (3.4%) *C. krusei*, 4 (1.9%) *C. dubliniensis*, other *Candida* spp. 11 (5.3%) (one *C. parapsilosis*, two *C. guilliermondi* , three *C. kefyer*, three *C. femata*, one *C. incanspiqua* and one *C.ciferi*) and unknown yeasts 10 (4.8%) were cultured on Sabouraud dextrose agar (Merck, Germany) and incubated at 37°C for 24 hours.

3.5. Preparing Yeast Suspensions

Yeast inoculums was prepared by picking two to three colonies of > 1 mm diameter from an overnight culture of *Candida* species, growing on Sabouraud dextrose agar at 35°C, and suspending them in 2 mL of 0.85% normal saline. The resulting suspension was vortexed for 15 seconds and this procedure yielded a yeast stock suspension of 1×10^6 - 5×10^6 cells/mL. A working suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which results in 0.5 - 2.5×10^3 cells/mL. The cell density was adjusted to 0.5 - 2.5×10^3 cells/mL.

3.6. Candida spp. Isolates Sensitivity to Caspofungin

Micro dilution plates were set up according to the NCCLS (CLSI) M27-A3 guidelines. Thermo Scientific™ Nunc™ MicroWell™ with 96-well flat-bottomed micro dilution panels is ideal for microscopic and optical measurements. Each micro plate had two drug free growth controls, one with the media alone (growth control) and the other with media containing an equivalent amount of solvent used to dissolve the drug (solvent control).

3.7. Minimum Inhibitory Concentration

Plates were incubated at 35°C and MICs were read visually after 48 hours using mirror. Rates of resistance were determined according to the MIC breakpoints proposed by NCCLS (CLSI) M27-A3. Caspofungin, an MIC of > 2 μg/mL, was used to identify caspofungin-non-susceptible *Candida* spp. (18). Fluconazole with the MICs of > 64 μg/mL, and 16 - 32 μg/mL were used to identify resistance, and susceptible dose-dependence against fluconazole, respectively (19). The growth level in each well was compared to that of the positive control. Antifungal activity was expressed as the MIC of each drug against the isolate. The following resistance breakpoints were used according to CLSI guidelines (17). MIC rates of caspofungin against *C. parapsilosis* ATCC 22019 were 1 and 0.0625 μg/mL, and against *C. krusei* ATCC 6258 were 0.5 and 0.5 μg/mL, respectively. MIC50 and MIC90 were also calculated. Data were analyzed by SPSS version 11.5.

Table 1. *In Vitro* Activity of Caspofungin Against Fluconazole-Resistant *Candida* spp. Clinical Isolates in Iran [a,b]

	Fluconazole-Resistant Isolates MIC ≥ 64	Caspofungin-Non Susceptible Isolates (MIC > 2) in Fluconazole-Resistant Isolates	Caspofungin-Susceptible Isolates (MIC ≤ 2) in Fluconazole-Resistant Isolates
***C.albicans* (108)**	8 (7.4)	1 (12.5)	7 (87.5)
***C. glabrata* (51)**	15 (29.4)	1 (6.6)	14 (93.4)
***C. tropical* (16)**	3 (18.7)	0	3 (100)
***C. krusei* (7)**	5 (71.4)	1 (20)	4 (80)
***C. dubliniensis* (4)**	0	0	0
Other *Candia* spp. (11)	0	0	0
Unknown yeasts (10)	2 (20)	0	2 (100)
Total (207)	33 (16)	3 (9)	30 (91)

[a] Data are presented as NO. (%).
[b] Other *Candida* spp. Includes: *C. kefyer* (3), *C. femata* (3), *C. guilliermondii* (2), *C. parapsilosis* (1), *C.ciferii* (1), *C.incanspiqua* (1).

4. Results

The most abundant species isolated from high-risk patients with cancer, AIDS and diabetes were *C. albicans* (52.2%), *C. glabrata* (24.6%), *C. tropicalis* (7.7%) and *C.krusei* (3.4%), respectively. The results revealed that 30 (91%) out of 33 fluconazole-resistant isolates of *Candida* spp. and other yeasts were susceptible to caspofungin. Only 3 (9%) out of 33 fluconazole-resistant isolates of *Candida* spp. were non-susceptible to caspofungin. The current study showed that *in vitro* caspofungin was more active against Iranian fluconazole-resistant *Candida* spp. and some other yeasts isolated from clinical samples in Iran ($P < 0.001$).

5. Discussion

Despite improvements in medical products, some health problems still remain unchanged (20, 21). Furthermore, innate or acquired antifungal resistance may pose a serious problem to antifungal treatment (20, 21). Antifungal resistance among invasive isolates of *Candida* is not common; therefore, it is still a concern, particularly for *C. glabrata* and *C. krusei* (22, 23), both of which are used to show intrinsic (*C. krusei*) or acquired (*C. glabrata*) resistance against fluconazole (24). Caspofungin is a fungicidal echinocandins and is active against many species of *Candida* (24-27). Caspofungin and other echinocandins exhibit potent activity against fluconazole-resistant *Candida* spp. (24, 26, 28-30). Pfaller et al. determined the *in vitro* activity of caspofungin against 351 fluconazole-resistant *Candida* isolates (24), and reported that 99% were susceptible to caspofungin at the MIC of 2 g/mL (24). Bachmann et al. reported that caspofungin was equally active against fluconazole-susceptible and fluconazole-resistant isolates (6). Lyon et al. evaluated the susceptibility of 5,821 isolates of *Candida* spp. They reported that *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. lusitaniae* were quite susceptible to fluconazole and *C. glabrata* was less susceptible to fluconazole (31).

Caspofungin exhibited (99.8%) significant activity against all species of *Candida* Lemos et al. reported 157 fluconazole-resistant *Candida* isolates out of 3959 *Candida* species isolated from clinical samples (2). The current study showed that caspofungin was active against the fluconazole- resistant *Candida* isolates (2). Silver et al. evaluated *in vitro* susceptibility of 80 *C. glabrata* species isolated from clinical samples to caspofungin (L). Their results showed that caspofungin had significant activity against fluconazole-resistant isolates, isolates susceptible and dose-dependent susceptible to fluconazole (32). Evaluated the activity of caspofungin against 3,959 isolates of *Candida* spp., and reported that a total of 157 isolates were resistant to fluconazole. Caspofungin showed the same activity against fluconazole-resistant isolates that it showed against isolates susceptible and dose-dependently susceptible to fluconazole (33). The current study determined *in vitro* activity of caspofungin against Iranian fluconazole-resistant *Candida* spp. isolated from clinical samples in Iran. Results showed that 30 (91%) out of 33 isolates were susceptible to caspofungin and 95% of the fluconazole-resistant, susceptible, and the growth of dose dependent *Candida* spp. isolates were inhibited by caspofungin; 7 (87.5%) out of 8 *C. albicans*, 14 (93.4%) out of 15 *C. glabrata* and 4 (80%) out of 5 *C. krusei* fluconazole-resistant isolates were inhibited by MIC ≤ 2 of caspofungin; the growth of 100% of *C. tropicalis* were inhibited by caspofungin. These findings confirm and extend the ones previously reported regarding significant activity of caspofungin (1, 2, 6, 24, 31-33).

Ortiz de la Tabla-Ducasse et al. in their *in vitro* study reported that caspofungin was very active against a variety of fluconazole-resistant *Candida* strains isolated from clinical cohort of HIV-infected patients. The MIC50 and MIC ranges of caspofungin against *C. albicans* were slightly higher than those of *C. glabrata*, which confirm the results of the current study (34). Posteraro et al. reported that their results represent further evidence for the excellent antifungal potency of caspofungin, particularly against *C. glabrata* isolates, expressing cross-resistance to azoles that confirms the results of the current study (8). The current *in vitro* study showed that caspofungin appears to be more effective against fluconazole-resistant *Candida* species and some other yeasts isolated from clinical samples in Iran.

Authors' Contributions

Conception, study design/provision, patients/sample collection and data assembling: Farideh Zaini, Mahin Safara, Parivash Kordbacheh, Hamideh Shekari Ebrahim Abad, Vida Mortezaee; data analysis and interpretation: Mahmoud Mahmoudi; Manuscript writing: Hamideh Shekari Ebrahim, Vida Mortezaee; Final Approval: Farideh Zaini.

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